

Development of a novel mosaic influenza antigen

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December 2015

- *Marianne Øygarden*

Abstract

The main problem with influenza is the lack of broadly protective vaccines. In order to be prepared against an emerging pandemic, we need to generate vaccines that can address the diversity of the influenza virus. Influenza strains diverge by 1-2 % each year, due to the always ongoing antigenic drift and the occasional antigenic shifts. As a result, the annual influenza vaccines have to undergo frequent formulation changes, in order to elicit protection against the circulating influenza viruses. Thus, development of novel vaccines that can protect against circulating viral antigenic diversity is needed.

This study describes the development and testing of two mosaic influenza antigens that were designed to confer broader protection against influenza. The mosaic antigens were inserted as antigenic units into previously described vaccine proteins designed to target antigens to different surface molecules on APCs. The mosaic genes were generated *in silico* using available hemagglutinin sequences collected from the ncbi database. One mosaic contained epitopes of the HA1 subtype only (HA¹), while the other mosaic contained epitopes from all available subtypes of HA (HA¹⁻¹⁷). The modified vaccine constructs were equipped with targeting units that would direct them to various receptors on APCs, such as chemokine receptors 1, 3 and 5, Xcr1, and MHC class II molecules. The ability of the vaccine constructs to mediate broad protection against influenza was tested by measuring the induction of cross-reactive antibody and T cell responses. Furthermore, the ability to mediate protection against a lethal dose of different H1 influenza viruses was investigated by viral challenge.

DNA immunization with vaccine plasmids encoding the mosaic antigens targeted towards various receptors on APC demonstrated induction of cross-subtype antibodies and antigen-specific T cells. Both the APC-targeted HA¹ and HA¹⁻¹⁷ mosaic antigens could confer protection against a challenge with influenza H1N1. The most interesting antigen in this respect is the HA¹⁻¹⁷ mosaic, as this is more distantly related to HA from H1N1 influenza viruses.

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1 Introduction

Vaccines is seen as one of the greatest triumphs in the history of public health. Many major human diseases, like smallpox, polio and measles, have been brought under control by vaccination programs and focused surveillance of the diseases[1]. Still, other diseases are more difficult to prevent by vaccination. One of these diseases is caused by the influenza virus, which diverge by 1-2 % each year due to both minor and more major changes in its outer glycoproteins[2]. The resulting species variation creates the need for a novel vaccine that can meet these challenges.

1.1 Influenza

1.1.1 Disease burden

Influenza is a contagious respiratory illness that mainly affects the nose, throat, bronchi, and occasionally the lungs. It is caused by the influenza virus, *Myxovirus influenzae*, that belongs to the *Orthomyxoviridae* family of RNA viruses with a viral envelope. People infected with the influenza virus will typically experience symptoms of the common cold, like severe aching, headaches, fatigue, and normally fever[3]. In addition, seasonal influenza has a significant economic impact on society, as it reduces productivity in both the work place and at home due to incapacitating illness, and because of the health-care costs associated with treatment. Normally, we recover from "the flu" from within a few days to less than two weeks, but for parts of the population the disease can have more severe consequences. For the very young, the elderly and people with serious medical conditions, seasonal influenza associated complications claims thousands of lives every year. Annually, seasonal influenza has been estimated to cause 250 000 – 500 000 deaths worldwide, and to inflict 3-5 million cases of severe illness[4].

In addition to the annual impact of seasonal influenza comes the potential social and economic burden of a pandemic influenza. With uneven intervals, reassortments between different strains of influenza may give rise to novel viruses against which the population has no prior immunity[5]. In such instances, an influenza pandemic may emerge. Although rare, the mortality rate associated with these large outbreaks can be striking, and even healthy

people might be at risk of complications[6]. The 1918-1919 influenza pandemic was the most devastating pandemic in modern history, claiming somewhere between 20 and 40 million lives. A disproportionate amount of the pandemic deaths was found in the young adult population[7]. In whatever part of the globe it struck, healthy young adults between 20 and 40 years of age were most severely hit by the pandemic[7]. A possible explanation for this unusual age distribution is cytokine storms, that are a potentially fatal immune reaction consisting of a positive feedback loop between cytokines and white blood cells, leading to highly elevated levels of various cytokines[8]. Thus, a healthy immune system can under certain circumstances, such as pandemic influenza, become uncontrollably activated, and thereby cause significant damage to tissue and organs[8].

1.1.2 Virus structure and hemagglutinin

The influenza virion has a roughly spherical shape and is characterized by an external layer with hundreds of spike-like projections. This outer layer is composed of lipids obtained from the last host cell, when the newly made virions budded from its surface. Inside the envelope is a coat formed by viral matrix proteins that bind both the envelope and the viral RNA genome simultaneously[9]. Each of the eight negative sense RNA molecules (segments) of the genome is complementary to one mRNA, and one mRNA encodes either one or two of the viral proteins. Segment 1-3 encodes the proteins Polymerase B2, Polymerase PB1 and Polymerase PA, respectively, which together make out the components of the virus' RNA polymerase. Segment 5 encodes Nucleoprotein (NP), a structural protein that encapsidates the viral RNA. Segment 7 encodes the matrix protein M1, which make out the inner coat between the envelope and the RNA-nucleoprotein core, and M2, an ion-channel integral of the viral envelope. Segment 8 encodes the non-structural proteins NS1 and NS2, and lastly, segment 4 and 6 encode the proteins hemagglutinin (HA) and neuraminidase (NA), respectively[9] (fig. 1.1). Both of these proteins are embedded in the viral envelope, but they have opposite functions. NA mediates the release of newly formed virions after replication by cleaving sialic acid residues from glycoproteins or glycolipids. HA's function is on the contrary to enable the virus to bind and fuse with target cells[10].

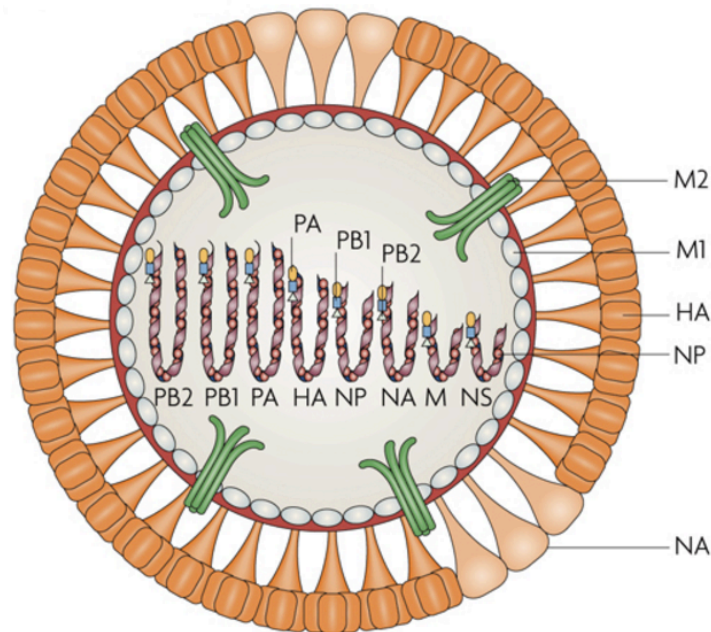


Figure 1.1. **Schematic of an influenza A virus:** The lipid envelope of the influenza virus is derived from the membrane of its host cell. The envelope proteins, HA, NA, and M2, are embedded in the lipid bilayer. HA and NA are the main surface glycoproteins of influenza A viruses. Figure reprinted from Subbarao (Subbarao 2007).

The rod-shaped hemagglutinin molecule is the most abundant protein on the surface of influenza. It is a trimer made up of identical subunits, where each is made up of two polypeptides resulting from a cleaved precursor. The molecule has a conserved stem region where the amino acid composition hardly vary among different strains, and a globular head region with a greater variety[10]. Before cleaving of the precursor, hemagglutinin exists as one long polypeptide, HA0, which gives the virus the ability to contact and bind target cells. The contact is mediated between the receptor-binding site at the tip of the molecule and sialic acids that are part of glycoproteins or glycolipids on the surface of cells[11].

When the influenza virus is bound to its target cell, hemagglutinin is cleaved into a state that activates its membrane fusion potential. The resulting polypeptides are called HA1 and HA2, and are held together by disulphide bonds[11]. The stem region of HA consists of parts of both HA1 and HA2, and contains a conserved stretch of 20 mostly hydrophobic residues at the N-terminus of HA2. This stretch is called the "fusion peptide", as it mediates the fusion process between the membrane of the virus and the membrane of the host cell[10]. Upon entry of influenza virus into lysosomes, the lowered pH will trigger a conformational change in hemagglutinin. This leads to exposure of the fusion peptide that was previously sequestered

in the interior of the structure, and removal of HA1 from the host cell membrane (fig. 1.2)[12]. The fusion peptide will insert into and perturb the target membrane, before initiating formation of lipidic fusion intermediates. These intermediate "stalks" will eventually lead to a hemifusion of the membranes that forms a fusion pore in the host cell membrane, enabling virus entrance[10].

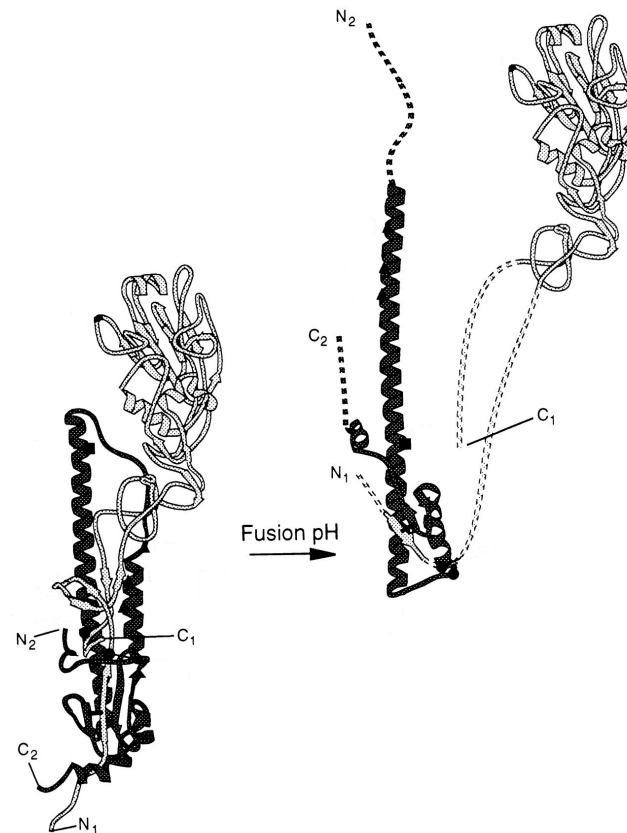


Figure 1.2. **Conformational change of influenza HA structure at the pH of membrane fusion:** The conformation of the HA monomer changes when entering an environment with a lower pH. The new positions of the carboxy- and amino termini of HA1 and HA2 (C1, N1, C2, and N2) make the fusion peptide at det N2 termini to "pop" out from the interior of the structure. This enables the fusion peptide to move towards the host endosomal membrane, and leads eventually to a fusion of the membranes. Figure adapted from Steinhauer (Steinhauer 1996).

The remaining part of HA1 makes out a globular domain at the tip of the hemagglutinin molecule, and three of these globular HA1 domains together make out the receptor-binding site. This part contains major antigenic epitopes which are targets for neutralizing antibodies[10]. As HA is also the most abundant protein on the cell surface, it is the primary target for a host cells immune response.

1.1.3 Influenza virus diversity

A major concern with the influenza virus is the great variety of influenza strains. They can be divided into three main types, the A, B and C type viruses, that can all infect humans, but that lead to varying degree of disease[4]. Type A viruses are the group responsible for large pandemics, and are further classified into subtypes based on the genes encoding the surface proteins hemagglutinin and neuraminidase. Both A and B type viruses lead to seasonal influenza epidemics every year, although the B type normally cause a less severe disease than the A type[13]. B type viruses are found only in humans and therefore have a less exchange of genetic information with other viruses, and might explain why they don't cause pandemics. Fewer viruses lead to less variation and opportunities for recombination with other virus strains. The C type causes only a mild respiratory illness and does not lead to pandemics[13]. The variety of the surface protein genes of type A influenza, has classified hemagglutinin into 18 different subtypes and neuraminidase into 11 different subtypes. The great number of possible gene combinations leads to a correspondingly large amount of different virus subtypes[13]. These subtypes are again divided into different virus strains, increasing the challenge of recognizing them with our immune system even further.

The replication of the RNA genome of influenza is relatively error-prone, and provides natural selection with many point mutations to act on. This ability to change its genes over time, or antigenic drift, leads to small changes in influenza virus epitopes, making our immune system unable to recognize them. Additionally, more abrupt and major changes in the genome, called antigenic shifts, also contribute to the evolution of influenza[14]. Antigenic shifts can take place when reassortment of genes from different viruses lead to generation of novel viruses. A lack of immunity against these viruses in the population, gives them the potential to cause pandemics. The H1N1/09 flu pandemic (swine flu) was caused by a new strain of influenza A virus of H1N1 subtype. This strain is thought to be a reassortment between four known strains of the influenza A virus H1N1 subtype, where one of them is endemic in humans, one is endemic in birds, and two are endemic in pigs[15]. The lack of pre-existing antibody-mediated immunity among the human population made it possible for the reassorted virus to spread rapidly. Although an estimated 284 500 people were killed by the disease, the pandemic was fortunately not as severe as initially predicted[16].

Antigenic shifts can also cause pandemic influenza in the absence of reassortment. An example to this is the scenario of a non-human novel influenza virus gaining the ability to transmit among humans in a sustained and efficient manner. The current global situation is characterized by an increase in animal influenza viruses co-circulating, and thereby exchanging genetic material[17, 18]. Viruses of the H5 and H7 subtypes are at present of greatest concern, as they can rapidly mutate from a form that causes mild symptoms in birds into a form that causes severe illness and death in poultry populations, resulting in devastating outbreaks[19]. Also, the death rates reported when people are infected with them, are strikingly high. The fear is that these highly pathogenic viruses might mutate into a form that gains the ability to infect between people as well[19].

1.1.4 Vaccination against influenza

Vaccination is the principal mean by which the substantial health burden of seasonal influenza can be reduced[20]. It reduces the likelihood of becoming ill, and of transmitting the disease to others. The seasonal influenza vaccines are usually trivalent vaccines, protecting against two influenza A viruses (an H1N1 virus and an H3N2 virus) and an influenza B virus. Quadrivalent vaccines protecting against two influenza A viruses and two influenza B viruses are also available, although more rarely used. The most common type of vaccine is the inactivated influenza vaccines (IIVs), containing inactivated virus, and that is available in many different versions. This type of vaccine has been available since the 1940s and is typically administered intramuscularly. The live attenuated vaccines (LAIVs) became available in the 1960s, and is administered as a nasal spray. They contain live attenuated (weakened) influenza virus, meaning that the viruses are still capable of replication but are not virulent [21].

The composition of both these influenza vaccines is based on the recommendations of the World Health Organization (WHO). The selection of viruses to be included is based on world-wide surveillance of influenza viruses circulating among humans, and is made almost a year ahead of the influenza season[22]. Despite all effort made to monitor the viruses, the match between viruses included in the vaccines and circulating viruses may vary significantly[23]. Even if they were to match, the ability of the influenza virus to continually undergo genetic changes (antigenic drift) leaves us with a vaccine that protects us for only a limited amount of time, and annual vaccination is therefore needed[24].

The currently available influenza vaccines are prepared by inoculating virus into chicken eggs. The fertilized hen's eggs are incubated for several days to allow the viruses to replicate. Fluid harvested from the eggs will contain high titers of virus ready to be either killed (inactivated) or weakened before they are purified[25]. The amount of chicken eggs needed for production of a vaccine is enormous. About 1-2 eggs is required for production of a single vaccine dose[26]. The process is also very time-consuming, and the time it will take to produce a novel vaccine will be almost a year. Another downside with this method, is the ability of influenza viruses to mutate into a form that is lethal to chicken embryos[26]. This could make it impossible to use eggs for production of vaccines against pandemic avian influenza viruses. Furthermore, the vaccines may not be suitable for people who are severely allergic (hypersensitive) to eggs, as there is a risk of allergic and anaphylactic reactions in response to them. For LAIVs, there is also a small risk that the attenuated virus may mutate and regain infectivity, possibly causing the disease instead of hindering it[27].

1.2 The immune system

The immune system is our defense against the numerous infectious organisms that our bodies are constantly exposed to. It comprises specialized organs, tissues and cells that all work together to prevent us from being infected[28]. This section will first address each of the two types of immunity, before the most important immune cells and their receptors are further described. Understanding how the immune system works is key to a successful vaccination that will generate a desired protection against influenza.

1.2.1 Innate immunity

The defenses of the innate immune system are activated immediately, or soon, after a pathogen is confronted. It is an ancient form of immunity which is genetically inherited and present in nearly all multicellular organisms[28]. The different elements of the system includes anatomical barriers, secretory molecules and cellular components[29].

Anatomical barriers

Only in rare cases does a pathogen succeed in penetrating the physical barriers of a body. The epithelial surfaces of the skin is impermeable to most invading pathogens and the mucosal

epithelium of the respiratory and gastrointestinal tract has a trapping effect[29]. Tears, saliva and nasal secretions contain chemical factors that can break down the cell walls of bacteria, and the pH of sweat will prevent them from growing[30]. However, tissue damage may occur, and an infection will follow as pathogens are able to penetrate the skin or mucosal surfaces.

Secretory molecules

A system of plasma proteins called the complement system is the second line of defense by the innate immune system. Its job is to complement immune cells in their work with clearing pathogens from the body, and performs this through a variety of antimicrobial functions[31]. Some specific complement proteins can by polymerization on cell surfaces lead to formation of pores in the membrane. This disrupts the lipid bilayer of the membrane and may lead to cell lysis[31]. Other complement proteins have proteolytic functions, and cleave antigens into peptides that signal the host cells to produce different chemical factors. The chemical factors, e.g. cytokines, will in turn enhance inflammation and immune responses to foreign antigens[31]. Other complement proteins have the ability to coat virions and thereby mark them for their destruction. Phagocytic cells with receptors for these coat proteins will be recruited to engulf and destroy the virus particles[31].

Cellular barriers

Macrophages are a type of myeloid cell that have the ability to phagocytose pathogens. They will engulf and digest foreign substances, microbes, cancer cells, and anything else that is not recognized as healthy body cells. In addition to phagocytosis, they are also capable of extracellular killing of infected self target cells[32]. Macrophages will recruit a different type of phagocytic cell, the neutrophils, that attack by completely surrounding the microorganism and digesting it with digestive enzymes[29]. Additionally, natural killer (NK) cells will migrate to the site of infection, and attack target cells by releasing cytotoxic proteins stored within secretory lysosomes[29]. Together, these cells make out the main line of defense of the non-specific immune system. All of them have germline-encoded pattern recognition receptors (PRRs) expressed on their surface. These receptors enable them, in an unspecific way, to recognize pathogen-associated molecular patterns (PAMPs), that are present on all pathogens. The patterns are made out of highly conserved molecules that pathogens share among them, but that host cells lack[33].

Lastly, the innate cells have a role in activation of the second type of immunity, mediated by the adaptive immune system. This takes place when macrophages degrade antigen into peptides and present them on their cell surface, leading to recruitment of the lymphocyte cells responsible for adaptive immunity[32].

1.2.2 Adaptive immunity

The innate immune system is able to fight off the vast majority of invading microorganisms, but a second more specific immune response is needed for the ones that escape it. A system of lymphocyte cells make out the adaptive, or acquired, immune system, that is present in all vertebrates[28]. This system uses an entirely different strategy for pathogen recognition, where each different cell has its own specific type of cell-surface receptor. In contrast to the germline-encoded PRRs of the innate system, these receptors are acquired through contact with specific pathogens during our lifetime[34].

The receptors can be made in an almost infinite number of versions due to genetic mechanisms that recombine the germline genes[35]. The result is a cell-surface receptor with a unique antigen specificity that enables a much stronger protection, which is important for keeping up with the rapidly evolving microorganisms[35]. After encountering their antigen, the lymphocytes proliferate to increase their numbers and undergo cellular differentiation to become effector cells[34]. The effector cells of adaptive immunity also mediate both a humoral response, like the secretory molecules of innate immunity, and a cell-mediated response, like the innate immune cells, against pathogens. The cells mediating the humoral response are called B lymphocytes, or B cells, whose function in the adaptive immune system is to secrete antibodies; a Y-shaped protein molecule used to identify and neutralize pathogens. The T lymphocytes, or T cells, play important roles in all arms of immunity and exist in two main types, the T helper cells and the cytotoxic T cells[34].

In addition a high specificity, the second advantage of adaptive immunity is the ability to create long-lasting protection[34]. Protection against a subsequent infection with the same pathogen is increased, due to a subset of B and T cells that have differentiated into memory B- and T cells. Upon re-exposure, the memory cells use their previous encounter with an antigen to beat it in a more effective way, preventing it from causing the disease. Because of this ability to "learn", we say the adaptive immune system has memory[35]. Immunological memory is used by vaccines in a similar way. Vaccines can induce an immune response

similar to that produced by a natural infection, without subjecting the recipient to the disease and its potential complications. The cells activated in response to vaccination can be retained long after the vaccine delivery.

1.2.3 T cells

T cells are a subset of lymphocyte cells defined by their development in the thymus and the T cell receptor (TCR) present on their cell surface. They migrate from their origin in the bone marrow for maturation and differentiation into various types of mature T cells in the thymus[36].

The T cell receptor is a disulfide-linked membrane-anchored heterodimeric protein made up of two polypeptide chains, normally of type α and β , and expressed as part of a complex with invariant CD3 molecules. For some few T cells, the polypeptide chains are made of two other variant polypeptide chains, the γ and δ chain. Each of the chains has a constant region, proximal to the cell membrane, and a variable region able to make contact with foreign antigens (fig.1.3)[37]. The TCR recognizes antigens as peptides in complex with certain protein molecules on the surface of antigen-presenting cells (APCs). APCs are cells that are able to process and present antigens on their surface[37]. The proteins are MHC (major histocompatibility complex) molecules, whose function is to bind peptide fragments derived from pathogens and display them on the cell surface.

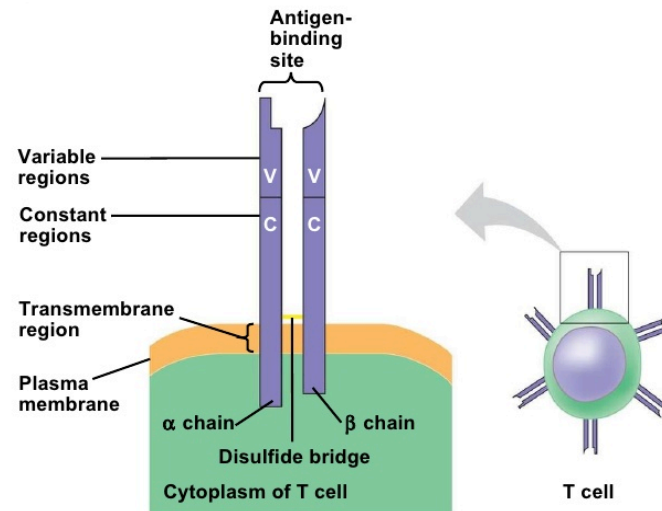


Figure 1.3. **Overview of a T cell Receptor on the surface of a T cell:** The TCR is a disulfide-linked membrane-anchored heterodimeric protein. It is composed of two different protein chains, normally the α and β chains, making out a heterodimer. Each chain consists of two extracellular domains; a constant (C) region and a variable (V) domain. The variable region of each polypeptide chain together make out the antigen-binding site. Copyright 2008 by Pearson Education, inc., publishing as Benjamin Cummings.

Before encounter with foreign antigens and activation, T cells go through two selection processes. The first process, positive selection, selects for cells that are capable of recognizing foreign peptides complexed with MHC molecules. The cells remaining after this selection end up being eliminated by apoptosis[38]. The other process, called negative selection, selects for cells that do not bind self-peptides complexed with a self MHC protein. To prevent the immune system from attacking cells that are part of the body, the self-reactive cells are also destined to die by apoptosis[36].

The T cells that have survived both selection processes, migrate to peripheral lymphoid organs where they are activated by APCs. The MHC molecules on these cells, presenting peptide antigens, interact simultaneously with the TCR and a co-receptor present on the T cell surface[37]. A complex of invariant transmembrane proteins (CD3) that is associated with the TCR, will transduce this binding of the peptide-MHC complex into intracellular signals. These will signal the cell nucleus to initiate transcription of genes needed for T-cell proliferation and differentiation into effector cells[38].

CD4 T cells

CD4 positive T cells carry the co-receptor protein CD4 on their surface. They mature into a type of effector cell called T helper cells, which have a major role in controlling and regulating the immune system by providing "help" to other white blood cells. They stimulate the immune responses of B cells, macrophages and cytotoxic T cells. However, they can only function after being activated themselves[36, 37].

MHC molecules exist in two forms that differ in both structure and function, and the type of MHC molecule that interacts with a T cell will depend on the type of co-receptor present on it. T cells with a CD4 co-receptor interact only with the MHC class II molecules, that have the ability to present foreign antigens found in extracellular fluid. APCs that carry MHC class II molecules are either dendritic cells, macrophages or certain B cells, which are the three cell types called professional APCs. Some activated T helper cells are involved with helping B cells secrete antibodies and are called T_H2 cells. The other group, the T_H1 cells, help macrophages destroy ingested microbes, and also help activate cytotoxic T cells, enabling them to kill their infected target cells[37].

CD8 T cells

T cells with a CD8 co-receptor present on their surface will interact with the MHC class I molecules. These molecules present antigens that have been taken up and degraded by the MHC class I-expressing cell. These cells can be virtually all nucleated cells, giving the CD8 positive T cells the possibility of interacting with a wide array of different cells. The CD8 effector T cells are cytotoxic cells capable of killing cells infected with an intracellular pathogen directly by strategies leading to apoptosis of the infected cell. Their ability to attack any variety of cell possible is of crucial importance, as all types of cells are potential targets for an intracellular pathogen[28, 36, 37].

1.2.4 B cells

B cells are small cells that originate in the bone marrow. They function in the humoral immunity component of the adaptive immune system by secretion of antibodies[39].

The B-cell receptor (BCR) is a transmembrane receptor protein located on the outer surface of B cells. It consists of a membrane-bound immunoglobulin molecule and associated $Ig\alpha$ and

Ig β heterodimers capable of signal transduction. The immunoglobulin contains a unique and randomly determined antigen-binding site that enables the receptor to specifically interact with just one type of antigen. The molecule is Y-shaped and consists of four polypeptide chains; two identical heavy chains and two identical light chains connected by disulfide bonds. Each heavy chain is made up of three constant domains and one variable domain, and each light chain is made up of one constant domain and one variable domain. One constant domain and one variable domain from each of the four chains make out the arms of the Y, which each contain one of two identical antigen-binding sites (fig. 1.4)[40].

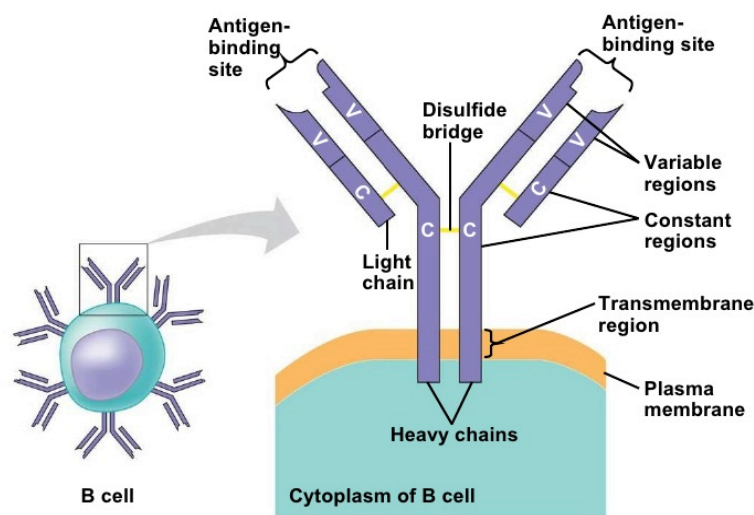


Figure 1.4. **Schematic drawing of typical antibody molecule on the surface of a B cell:** The antibody molecule is put together by four polypeptide chains. The two identical heavy chains make out the stem and the hinge region. The N-terminal parts of a heavy and light chain together make out an antigen-binding site. The two antigen-binding sites are identical. Copyright 2008 by Pearson Education, inc., publishing as Benjamin Cummings.

When the BCR interacts with membrane-bound antigen, a synapse is formed between the B cell and the surface of the pathogen. The transmembrane complex that is non-covalently associated with the membrane-bound antibody, containing intracellular immunoreceptor tyrosine-based activation motifs (ITAMs), will be activated by phosphorylation[39]. This creates a signaling cascade that leads to activation of genes associated with B cell activation. In addition to these signals, most B-cells need additional signals from an antigen-specific helper T-cell to become activated[39].

After activation, and subsequent encounter with a matching antigen, the B-cell will multiply to create an army of identical cells that all recognize the same pathogen. This takes place through a process called clonal selection[39]. At the same time, the cells will differentiate into a state where the immunoglobulin part of the BCR is secreted in large amounts, instead of being attached to the cell surface[39]. The secreted immunoglobulins, or antibodies, will bind pathogens and either neutralize them, or prepare them for uptake and destruction by phagocytes[40].

Neutralizing antibodies have the ability to prevent an infection, and mediate this by sticking to antigens on the surface of a pathogen and thereby immobilizing it. At present, the production of neutralizing antibodies against an infectious agent is the main aim of most vaccines. Antibodies can also, while bound to antigens with their antigen-binding region, bind to receptors on phagocytic cells with their Fc region, and thus serve as molecular adaptors. This will encourage white blood cells or macrophages to phagocytose, or eat, the pathogen. Having clumps of antibodies on the surface of a pathogen will serve as a signal to the body that intruders are present, and can thus recruit various types of other immune cells as well. The result is either cytolysis, where proteins of the complement system lyse the cell, or cytotoxicity mediated by various effector cells[39, 40].

1.3 APC-targeted DNA vaccines against influenza

This section will focus on how the concept of DNA-based immunization can be used to create novel vaccines against influenza that are targeted to specific cell types in the body. An explanation of the DNA vaccination strategy will be presented, before its advantages and disadvantages concerning influenza prevention are discussed. Further, one of the strategies that can be applied for improved immunogenicity of DNA vaccines, is explained. Lastly, a vaccine strategy that enables the delivery of APC-targeted influenza vaccines will be introduced.

1.3.1 DNA vaccines

DNA vaccination is the use of DNA plasmids to induce immune responses by direct injection into animals of DNA encoding antigenic proteins[41, 42]. The DNA will be introduced into appropriate tissues, and the cells present will start production of the antigen to which an immune response is sought[41, 42]. Immunization with this "naked" DNA may be able to protect against human disease without many of the disadvantages associated with current vaccines, such as long production-time, low vaccine stability, and the use of infectious agents[43]. Although approved for use in some animals, no DNA vaccines are available for use in humans as yet.

DNA vaccines can be rapidly constructed and manufactured, and could therefore offer a huge advantage should a pandemic influenza emerge[44]. Compared to generation of conventional influenza vaccines, DNA vaccines against influenza do not need to be grown in chicken eggs, which shortens the generation time drastically. A shorter production time will also solve the mismatch problem in annual influenza vaccines, between strains to be included in the vaccines, and the actual circulating strains. The stability of DNA reduce the costs of manufacturing DNA vaccines as they can easily be stored and distributed[43]. This enables a large-scale manufacture of vaccines which again could potentially enable vaccination on a global scale. This would mean that a vaccine could be available for people in developing countries aswell, where they are needed the most. Use of plasmids offers a conceptually safer strategy for vaccination, as they are non-live and non-replicating, leaving little risk for secondary infection. Also, the genes can be produced without exposure to live pathogens and with high fidelity to the wild type proteins.

A potential disadvantage with DNA vaccines is the concerns regarding whether the plasmid DNA can be integrated into genomes following vaccination[44]. This could potentially result in mutations and problems with replication of the host cell DNA, or result in chromosomal instability through the induction of chromosomal breaks or rearrangements. Another concern is that the inserted DNA might lead to insertional mutagenesis, by activation of oncogenes or the inactivation of tumor-supression genes[45]. Nonetheless, clinical trials with DNA vaccination applied in humans have investigated these concerns extensively and has so far found little evidence of integration[46, 47]. A second concern regards the potential of a DNA vaccine to induce anti-DNA immune responses, which would defeat the point of a vaccine as

a whole[44]. Research has as yet not reported immune responses against the introduced DNA[44, 48]. Based on this research, the main problem with DNA vaccines is not these slight risks, but instead their low immunogenicity[49, 50]. The low immunogenicity is hypothesized to stem, in part, from inefficient uptake of the plasmids by cells[45].

1.3.2 APC-targeting

Since the discovery of DNA vaccination, several advancements in antigen design, improved formulations, inclusion of molecular adjuvants, and physical methods of delivery have been made to improve immunogenicity[45]. One of the strategies that previously has shown to significantly increase immune responses after DNA vaccination, is targeting of the antigen to APCs[51]. Novel vaccine molecules have been generated that target antigen to APCs via a targeting unit directed against receptors expressed on their surface. The rationale behind this, is to increase antigen uptake, and the subsequent presentation of peptides to CD4 and CD8 T cells. Cells transfected with plasmid DNA encoding the vaccine molecules will secrete the encoded vaccine proteins that are then targeted to different surface molecules on APCs (fig.1.5). After binding to surface molecules, the vaccine proteins are processed by the APCs, and peptides from the antigen unit is presented on either MHC class I or MHC class II molecules on the cell surface[52]. Targeting to different surface molecules will lead to different immune responses, depending on which type of MHC molecule that ends up presenting the antigen peptides[52].

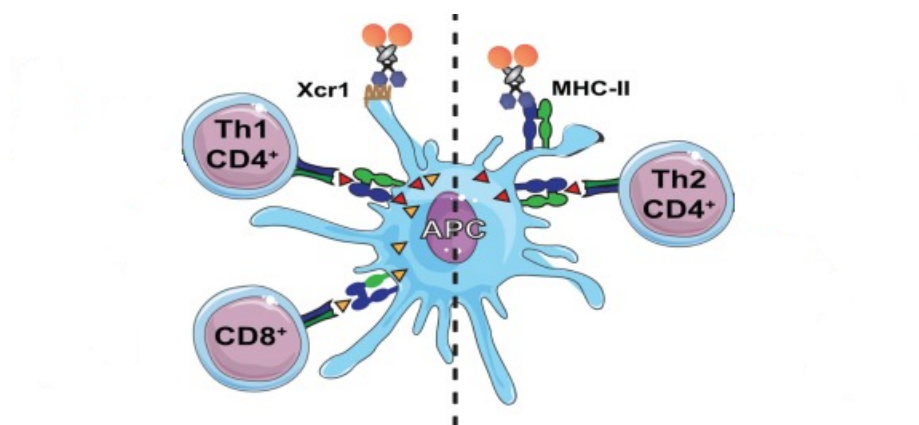


Figure 1.5: **Secreted fusion proteins have the ability to target antigen-presenting cells by binding to surface molecules:** The APC-targeted fusion proteins can be targeted to surface molecules on APCs and induce either B- or T cell responses. The fusion proteins are processed by the APC and peptides from the antigenic unit are presented on MHC I/MHC II molecules of the APC. Adapted from Grødeland et.al. (Grødeland 2015).

1.3.3 Vaccibodies

A vaccibody is a targeted immunoglobulin-based homodimeric DNA vaccine that can be used to target antigens to various surface molecules on APCs[53]. The vaccibody homodimer (fig.1.6) are made out of three modules, each of them serving a purpose to increase efficacy. Two individual identical amino acid chains make out the homodimer[53]. Each of them has an N-terminal targeting unit, a dimerization unit composed of a shortened human Ig hinge and a C_H3 domain from a human IgG, and an antigenic unit[51]. The targeting unit ensures the ability to attract and target APCs, which leads to an effective immune response. Binding to surface receptors will lead to a rapid internalization of the vaccibody and mediate maturation of the APC. Overall, this will enhance presentation of the antigenic unit to T cells[54]. The dimerization unit serves to connect the antigenic unit with the targeting unit, and also to facilitate the dimerization of the two monomeric proteins into a homodimer, enabling bivalency[51]. Any intact protein antigen can be used in the antigenic unit, which, depending on the selected targeting unit, can stimulate both B- and T cell immunity[52].

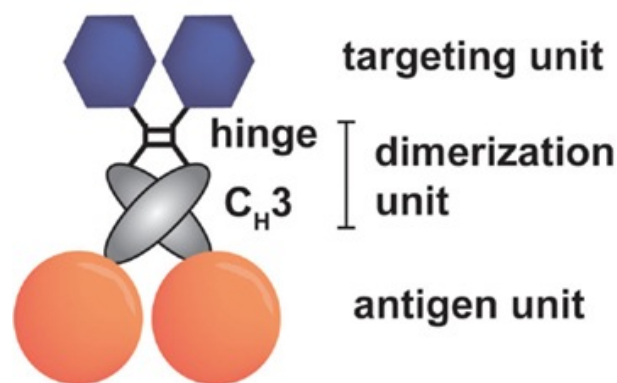


Figure 1.6. **Overall structure of the vaccibody molecule:** The vaccibody is a homodimer where each chain is composed of an amino-terminal targeting unit that binds surface molecules on APCs, a dimerization unit composed of a shortened human hinge and C_H3 domain, and an antigenic unit composed of the relevant antigen. Adapted from Grødeland (Grødeland 2015).

1.4 Universal vaccines against influenza

Current influenza vaccines primarily induce strain specific neutralizing antibodies[55]. Thus, vaccines against seasonal influenza have to be annually updated due to antigenic drift, and the vaccines are likely to offer no increased protection in the event of a novel influenza pandemic. Novel influenza vaccines that could offer broader protection are therefore important. The ultimate goal would be development of a vaccine that could be delivered once, and yet confer lifelong protection against influenza.

A broad protection against influenza can be conferred by both broadly neutralizing antibodies and broadly protective T cells. The vaccine-induced antibodies will typically be specific for the globular head of HA. However, the globular head is highly prone to mutations, rendering the antibodies inefficient against next year's influenza[56]. The stalk of HA is more conserved, but unfortunately also low immunogenic. In recent years, several studies have demonstrated such antibodies to be broadly protective[56, 57], but vaccine development using the stem as antigen has proven cumbersome[58, 59].

T cells typically bind epitopes that are fairly conserved within a subtype of influenza, and can as such offer broader protection against influenza than antibodies[60]. However, T cells cannot block the viral entry of influenza virus, but they can greatly reduce disease burdens and confer protection against increased morbidity and mortality[61]. Thus, vaccines aiming at the induction of protective T cell responses could prove very useful for protection of the population against influenza. Below, several strategies for vaccines aiming at the induction of T cells will be introduced.

1.4.1 Broadly protective vaccine antigens

Most universal vaccine design approaches currently focus on generating cross-protective cellular immunity. One such strategy is to identify conserved sequences, which are similar sequences that occur across species, and use these as vaccine antigens ([57, 62]). T cell epitopes are commonly highly conserved on internal proteins between different strains of influenza viruses[60]. One example is the nucleoprotein (NP), which is a major target for immunodominant CD8 T cell responses during an influenza infection. NP has been shown to induce effective immune responses and protection against various influenza A subtypes[63].

Another strategy is to construct a consensus sequence, where the “average” of many hemagglutinin sequences are combined into one gene. One study with influenza involved using many H5N1 hemagglutinin sequences, in order to generate a codon-optimized gene that could generate a broad protection. The vaccine managed to elicit robust cross-protective immune responses in mice against divergent H5N1 influenza viruses[64].

1.4.2 Mosaic vaccine antigens

Recently, a novel mosaic antigen approach has been shown to induce T cell reactivity against a broader variety of epitopes as compared to other approaches[65]. In this method, a genetic algorithm is utilized in order to computationally design sequences that are similar to natural sequences, but that maximizes the coverage of potential T cell epitopes[66]. The strategy was first applied for development of novel HIV vaccines, which, as influenza, is a virus that diverges from year to year[65, 67, 68]. Co-circulating HIV strains differ from one another by 20% or more in relatively conserved proteins and by up to 35% in their envelope protein, and the result is that each person infected with HIV have different versions of the virus[69].

For designing an effective HIV vaccine that can make the immune system recognize the highly variable HIV antigens, mosaic sequences were generated that were optimized to include the maximum number of potential T-cell epitopes from a set of viral proteins and that showed better coverage than consensus sequences made from the same set of viral proteins[66]. The mosaic sequence has been put together using gene fragments from many different viruses, and encodes in sum a mosaic protein where conserved regions are favoured. The resulting recombinant protein will consist of peptides of a certain length (k -mers) that can all be found in the set of input sequences, as opposed to a consensus sequence, which is made out of the most frequent residue at each position[70]. The mosaics both resemble natural proteins and contain only natural fragments, and so intracellular processing and presentation on MHC molecules will resemble the processing of a natural pathogen[70], which might explain the broader protection compared to the consensus. Quite recently, the mosaic strategy was demonstrated to be effective also in the context of influenza[2].

1.5 Aims of the study

As influenza viruses diverge by 1-2% each year[2], there is a need for novel influenza vaccines that can confer a broader protection against influenza. In this study, mosaic antigens were made using HA sequences from a collection of diverse influenza viruses. HA is the logical choice of antigen, as it is the most abundant surface molecule of influenza, and because it is responsible for the initial contact and fusion with influenza viruses. The mosaic HA genes will contain only natural T cell epitopes that were all present in the collection of HA sequences. The epitopes stimulating T cell responses will hopefully be the same epitopes that are processed and presented in natural influenza infections.

The mosaic HA sequences were applied as antigenic units of vaccibodies. Recent experiments have shown that the choice of targeting unit can promote the formation of either dominant antibody responses or T cell responses[71]. The vaccibodies were therefore targeted to different surface molecules: the chemokine receptors CCR1, CCR3 or CCR5 by MIP1 α , the chemokine receptor Xcr1, by XCL1, and to MHC class II molecules by a single chain variable fragment (scFv). Vaccibodies targeting the hapten NIP were constructed as a non-targeted control.

The aims of the study were to:

1. Construct mosaic hemagglutinin antigens to be used as antigenic unit of Vaccibodies
2. Construct Vaccibodies containing the modified hemagglutinin antigens
3. Analyze the *in vitro* structural and functional properties of the novel vaccine proteins
4. Investigate the vaccines' ability to induce antigen-specific antibodies and T cell responses
5. Examine influenza protection in mice following vaccination

2. Materials and methods

This section will first describe the bioinformatic methods used for generation of the mosaic hemagglutinin antigens. Further, the laboratory techniques applied for construction of the novel vaccine proteins are listed. Lastly, the different techniques applied in animal experiments are presented. An introduction to each technique is briefly described prior to the following procedures. Where commercial kits were used, the supplied protocols from the manufacturer are referred to.

2.1 Bioinformatic methods

Before generation of the mosaic hemagglutinin genes, a total of 387 HA sequences were collected from the National Center for Biotechnology Information (NCBI) protein database. All available sequences that were complete and non-redundant were collected. The sequences collected represented all classes of HA except subgroup 18, for which there were no complete sequences. The optimized mosaic sequences were generated by a genetic algorithm using the Mosaic Vaccine Designer tool [72], and synthesized commercially (Genscript, USA).

2.1.1 Building phylogenetic trees

Phylogenetics is the study of evolutionary relatedness among organisms. Building of a phylogenetic tree can be used as a method to represent the genetic relationship between species. Before generation of phylogenetic trees for the HA subtypes, multiple alignments were performed using COBALT (Constraint-Based Multiple Alignment Tool[73]). The tool computes an alignment using conserved domain and local sequence similarity information. The alignment is then utilized for building of a tree that will visualize similarities between the sequences. This was performed using the Phylogenetic Tree operation in COBALT.

Phylogenetic trees were built for subgroups of HA where numerous HA sequences were available for collection, in order to get an overview of the genetic relations between the HA sequences in each subgroup. A selection of input sequences, sequences that was put into the

Vaccine Designer for generation of a mosaic, could then be chosen from the tree. Generation of phylogenetic trees were necessary for the subgroups H1, H2, H4, H5, H6, H7, and H9. For the remaining subgroups where few sequences were available, all collected sequences were used as input sequences.

For generation of a mosaic based on only the HA1 subtype, (HA¹), the selection of input sequences was made from the 167 sequences of the H1 subtype. In addition to the genetic similarity of the sequences, the year and location where the HA sequence was first detected, were also taken into account when selecting input sequences. A set of 18 H1 sequences were chosen for generation of HA¹ with the Mosaic Vaccine Designer tool. The second mosaic, HA¹⁻¹⁷, was constructed from a total of 112 HA sequences representing all 17 available HA subtypes. Phylogenetic trees were produced in the same way as for the H1 subgroup, and sets of input sequences from the H2, H4, H5, H6, H7, and H9 subgroups were chosen based on the same criteria. Together with these, all available sequences from the remaining H3, H8, H10, H11, H12, H13, H14, H15, H16, and H17 subgroups were also used as input sequences when generating the HA¹⁻¹⁷ mosaic (table in Appendix 4).

2.1.2 Mosaic Vaccine Designer

The Mosaic Vaccine Designer tool uses the recently published "mosaic" method to generate protein sequences that are optimized for high-frequency *k*-mers. It utilizes a genetic algorithm for optimizing of a vaccine antigen made from a population of artificial recombinants (fig. 2.1). The recombined sequence population is generated using random two-point recombination of natural sequences in an input set. Four sequences are picked from the population (or one of them is picked from the natural input sequences), and the two "better of pair" sequences are recombined in order to generate a "child" sequence. Both the child and the parent sequences are scored by computing the coverage of *k*-mers. The coverage is defined as the mean fraction of natural-sequence *k*-mers included in the sequence, averaged over all natural sequences in the input data set. If the sequence coverage of the child exceeds that of any of the four randomly picked sequences, the lowest-scoring one is replaced with the child. This cycle of child generation is continued until improvement of *k*-mer coverage in the population ceases. The child with the highest score of epitope coverage will then be selected as the populations best representative and resulting sequence generated by the algorithm[70].

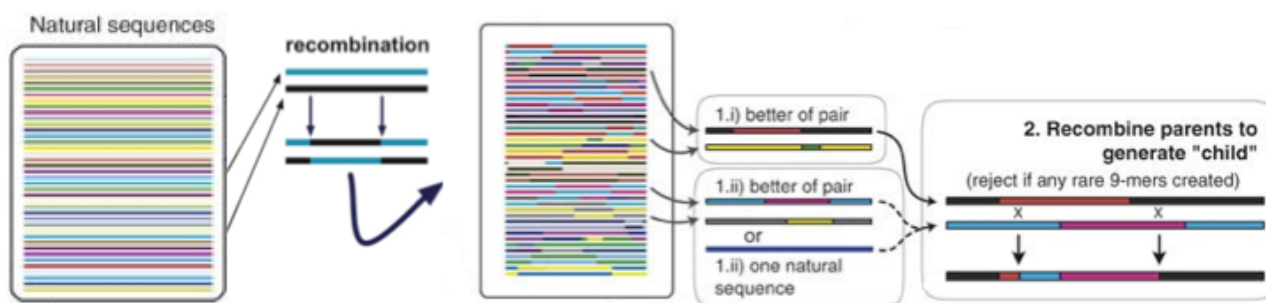


Figure 2.1. **Schematic of how the mosaic vaccine designer generates mosaics:** The mosaic vaccine designer generates mosaics from a set of natural input sequences that are recombined into recombined sequence populations. The recombined sequence populations evolve for improved epitope coverage by choosing two parent sequences that are recombined for generation of a child sequence. Figure adapted from Fischer (Fischer 2007).

A number of parameters had to be set in the parameter options of the vaccine designer tool. Most importantly, the epitope length was set to 9 amino acids in an attempt to select for epitopes that are presented by both MHC class I and class II molecules, and thereby capture of both CD8 T cell epitopes and CD4 T cell epitopes. The cocktail size was set to 1 in order to generate a single peptide that represented all uploaded sequences. As the input set of sequences were relatively small, the rare threshold was set to the default value 1. When rare threshold is set to 1, every sequence with a length of 9 amino acids (9-mer) that exists in the input set will count in the score. These parameter settings will be further discussed in section 4.1.1. The 18 H1 sequences were uploaded for generation of the HA¹ mosaic, and the 112 sequences collected from all available subgroups were uploaded for generation of the HA¹⁻¹⁷ mosaic. The resulting mosaic sequences are shown in Appendix 4: Results.

2.1.3 Sequence alignments using BLAST

Alignment of protein sequences was performed using Basic Local Alignment Tool (BLAST)[74]. BLAST is an algorithm for comparing sequence information and will find regions of local similarity between two sequences. The resulting alignment will display the differences in amino acid composition between sequences.

2.1.4 Coverage assessment using Epicover

The Epitope Coverage Assessment Tool[75] facilitates coverage comparisons for potential antigens. Coverage is defined as the mean fraction of natural-sequence 9-mers included in the antigen, averaged over all natural sequences in the input data set. The potential epitope cover by an antigen is calculated using the optimization metric used by the Mosaic Vaccine Designer tool. The tool calculates the fraction of 9-mers per sequence in the input set that are covered by the antigen. Both exact matches and near-matches contribute to the coverage of epitopes, due to the fact that similar epitopes may cross-react. In this study, both the off-by-1 amino acid matches and the off-by-2 amino acid matches were included in the calculation of coverage.

2.2 Cloning techniques

Standard molecular biology techniques were used for construction of the novel vaccine proteins. The mosaic genes were delivered from Genscript in pUC57 vectors with flanking SfiI sites, and were subcloned into a pLNOH2 expression vector, using the corresponding SfiI restrictions sites.

2.2.1 Restriction digest

Restriction enzymes are naturally occurring enzymes able to cleave DNA molecules at their specific cleaving sites. In this study, a restriction digest was performed in order to cleave the HA sequences from the pLNOH2 and pUC57 vectors. The cleaving was performed with the restriction enzyme SfiI (BioNordika, NEB), which will cleave DNA at the sequence GGCCNNNN↓NGGCC, where N is any base and ↓ is the point of cleavage. The restriction digests were performed according to the following protocol.

Protocol

- In a 1,5 ml tube, combine the following:
 - 8 µl of plasmid DNA
 - 2 µl BSA
 - 2 µl NEB4

- 1 μ l of SfiI
- 7 μ l of dH₂O (= total volume of 20 μ l)
- Mix gently by pipetting.
- Incubate tube at 50 °C for 1 hour, according to the manufacturer's instructions.

2.2.2 Agarose gel electrophoresis

For separation of fragments from the digested DNA, a gel electrophoresis was performed. In a gel electrophoresis, DNA fragments is separated according to size due to an electric current pulling them through a gel. The fragments will wander towards the positive cathode of the electrophoresis chamber due to the negatively charged phosphate backbone of DNA. The gel consists of a matrix of agarose, a polysaccharide polymer material extracted from seaweed. As the DNA moves through the matrix, the larger fragments are more likely to be impeded and slowed down by the gel, enabling separation of different sized molecules. In this experiment, a 2% agarose gel was cast according to the protocol in Appendix 2. A molecular weight standard was added to the gel in order to estimate the sizes of the different fragments. The fragment sizes was deduced by comparing bands from the respective samples with the bands of known sizes in the ladder. The bands corresponding to the appropriate fragment sizes could then be excised from the gel using a clean scalpel and stored.

Gel extraction

A gel extraction was performed in order to isolate the DNA fragments in the stored gel pieces. The isolation procedure was performed with a QIAquick Gel Extraction Kit (QIAGEN) and in accordance to the manufacturer's protocol.

2.2.3 Ligation

For insertion of the mosaic HA antigens into the pLNOH2 vector, a ligation reaction was set up. The reaction was catalyzed by T4 DNA ligase (Roche), which is an enzyme isolated from bacteriophage T4. T4 DNA ligase is able to catalyze the formation of a phosphodiester bond between neighboring 3'-hydroxyl- and 5'-phosphate ends in doublestranded DNA. As a result, a covalent bond was formed between the DNA sequence encoding HA and the DNA sequence of the vector. The reaction was set up according to the following protocol.

Protocol

- In a 1,5 ml tube, combine the following:
 - 1 μ l of T4 DNA ligase (added last)
 - 2 μ l of vector DNA (vaccibody)
 - 7 μ l of insert DNA (mosaic)
 - 2 μ l 10 x Ligation Buffer (Roche)
 - 8 μ l nuclease-free water (= a total of 20 μ l)
- Mix all components by pipetting up and down and centrifuge briefly.
- Incubate over night at 3-4 °C.

2.2.4 Transformation

The ligated plasmids were transformed into chemically competent cells called TOP10 cells (Invitrogen). These are chemically competent E.coli cells that are ideal for high-efficiency cloning and plasmid propagation. Transformation is the process by which foreign DNA is introduced into a cell. Transformation of TOP10 cells were used as the means of replicating the ligated pLNOH2 plasmids, in order to increase the amount of DNA. This was possible because the pLNOH2 vector contains a bacterial origin of replication, in addition to the mammalian origin. It also carries an ampicillin resistance gene, enabling positive selection of the bacteria that has been successfully transformed. Only cells containing the plasmid were able to grow and form colonies when plated onto agar plates containing ampicillin.

Protocol

- Take the competent TOP10 cells out of -80 °C and thaw on ice (approximately 10-15 min).
- Mix 1-2 μ l of ligation reaction into one tube of competent TOP10 cells.
- Place the cells/DNA mixture on ice for 20-30 minutes.
- Heat-shock the transformation tubes by placing them in a 42 °C heating block for exactly 45 seconds.
- Put the tubes back on ice for a few minutes.
- Add 500 μ l of 1xLB media and grow in a 37 °C heat block for 45 minutes.
- Centrifuge the tubes at 3000 rpm for 3 minutes to pellet the bacteria.

- Discard media until ~ 100 µl is left and resuspend the bacterial pellet.
- Plate the transformation onto agar plates containing ampicillin.
- Incubate plates over night at 37°C.

Isolation of DNA

The plasmids were isolated from the bacterial cells using a small-scale isolation technique (miniprep). The Wizard Plus SV Miniprep kit (Promega) applied, are able to yield a concentration of 50-100 µg of DNA. The procedure was performed according to the manufacturer's protocol.

2.2.5 Diagnostic restriction digest

A diagnostic restriction digest was performed by cutting the plasmid into specifically sized pieces prior to analysis of the resulting fragments using gel electrophoresis. The procedure was performed in order to verify the presence of the inserted fragment, encoding the mosaic HA, in the pLNOH vector. The plasmid was cut using enzymes with restriction sites both inside and outside of the inserted fragment. By running the cut plasmids on a gel, the sizes of the fragments, and thereby the presence of the insert, can be deduced. The restriction enzyme BsmI (BioNordika, NEB), has a cut site 585 bp into the HA¹ mosaic and was used to cut the plasmids containing it. The enzyme will cleave at the DNA sequence G↓GATCC, where N is any base and ↓ is the point of cleavage. The plasmids containing HA¹⁻¹⁷ were cut with BamHI (BioNordika, NEB), which cuts at 515 bp into the inserted mosaic fragment, at the DNA sequence GAATG↓C. The restriction was performed according to the protocol in section 2.2.1.

2.2.6 DNA sequencing

DNA sequencing was performed in order to determine the precise order of nucleotides in the vaccine plasmids. This was done as an additional test to verify that the vaccibody constructs contain the correct HA antigen and targeting unit. The method used for sequencing is the Sanger method, which involves *in vitro* synthesis of the DNA to be sequenced. The sequencing was performed by GATC Biotech (GATC, Germany). Purified plasmids were premixed with primer and shipped in 1,5 ml tubes. The three different sequencing primers applied were diluted to 5 uM before use. The 5' pLNO primer initiates synthesis of the

targeting unit from the 5'. The 3'C pLNOH2 will start sequencing from the 3' end of the inserted fragment. A third primer, GATC-5'hlhinge-115298, will initiate sequencing from the 5' of the hinge region. The DNA sequences of the primers are listed in table 1.

Table 1. Primers used in the sequencing of ligated vaccine plasmids and their DNA sequences

Primer	Sequence
5' pLNO	TCA CAG TAG CAG GCT TG
3'C pLNOH2	ATG GCT GGC AAC TAG AAG
GATC-5'hlhinge-115298	GAGAGGGTCTTCTGGCTT

After receiving the sequencing results, *in vitro* sequences were aligned with the known sequences of the mosaic genes and targeting units. A high match between the sequences verify that the cloning of vaccine constructs has been successful.

2.3 Sandwich ELISA

The enzyme-linked immunosorbent assay (ELISA) is a test that utilizes antibodies and color change to identify a substance. Performing an ELISA involves at least one antibody with specificity for a particular antigen and usually a polystyrene microtiter plate used as a solid support. In a sandwich ELISA, a capture antibody is attached to the microtiter plate before addition of the antigen. A blocking buffer is added to prevent unspecific binding of antibodies to the wells. The proteins in the blocking solution will bind the area of the well not occupied by the capture antibody. The samples containing the antigen can then be added to the wells. Addition of a specific biotinylated detection antibody will trap, or "sandwich", the antigen in between the two antibodies (fig. 2.2). For detection, an enzyme-linked secondary antibody that binds non-specifically to the primary antibody is added. Addition of the enzyme substrate will create a color signal that gives quantitative information about the antigen.

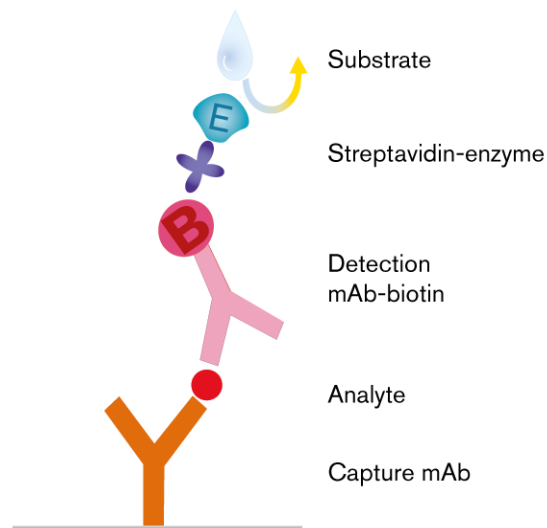


Figure 2.2. **Overview of the Sandwich ELISA format:** The antigen is bound between two primary antibodies; a capture antibody and a biotinylated detection antibody. The antigen is detected indirectly by an enzyme-linked secondary antibody. Addition of the enzyme substrate creates a color signal. Copyright 2015 by Mabtech [76]

Protocol

- Add 100 μL of capture antibody (diluted in PBS azid) to each well, cover the plate, and incubate ON at 4°C .
- Add 200 μL of blocking buffer (recipe in Appendix 3) to each well, cover the plate, and incubate at RT for 1 hour.
- Thoroughly decant solution from wells and wash 3 times with ELISA wash buffer (recipe in Appendix 3).
- Add samples (SN or serum) and perform appropriate dilutions in ELISA buffer (recipe in appendix 3).
- Cover plate and incubate for 2 hours at RT.
- Thoroughly decant solution from wells and wash as before.
- Add 100 μL detection antibody to each well, cover the plate, and incubate for 1 – 1½ hour at RT.
- Thoroughly decant solution from wells and wash as before.
- Add 100 μL of diluted strep-ALP (GE Healthcare) (1:3000 in ELISA-buffer) to each well, cover the plate, and incubate for 40 minutes at RT.
- Prepare 1 mg/ml of phosphatase substrate in substrate buffer (recipe in appendix 3).

- Add 100 μ L of substrate solution (1 mg/ ml) to each well and develop until wells turn yellow.
- Read the absorbance at 405 nm with an ELISA plate reader at appropriate time points.

2.3.1 ELISA for detection of vaccine proteins

Three different ELISA assays were in this study applied for examinations of proper structure and function of the vaccine proteins. Supernatant was collected from transiently transfected HEK293E cells. The cells were grown in complete tissue culture medium: RPMI-1640 containing 10% FCS and supplements (ThermoFischer). 1 μ g of DNA was used per well of a 24 well flat bottom cell culture plate (Corning Costar cell culture plate, Sigma-Aldrich). Each well was seeded with 2×10^5 cells and transfected the next day. Supernatants for use in sandwich ELISA were collected after 3 and 7 days. All ELISA assays were performed following the above protocol.

Test of secretion of vaccine constructs in vitro

In this assay, microtiter plates were coated with MCA878 (1:1000) (Bio-Rad), an anti-human IgG specific for the dimerization unit of the vaccibodies, and detection was performed using a mAb specific for the Fc-region of human IgG (1:1000) (HP6017-bio, Sigma-Aldrich). Both antibodies bind the dimerization unit of the vaccibodies. Supernatant containing α MHCII-HA^{PR8} was used as a positive control, and supernatant with no DNA added was used as a negative control (mock).

Test for presence of correct targeting unit in MIP1 α - and α NIP-targeted vaccines

In a second ELISA assay, two microtiter plates were coated with either hCCL3/MIP1 α antibody (1:1000) (R&D Systems), or NIP-BSA, in order to verify the presence of the α NIP - and MIP1 α targeting units. All eight constructs and a mock were added to both plates along with positive controls containing the targeting unit used as coat (either α NIP^{pos} or MIP1 α ^{pos}). Detection was performed with HP6017-bio (1:1000).

Test of the vaccine constructs' binding capacities

Two microtiter plates were coated with the capture antibody MCA878 (1:1000) and supernatant containing each of the eight constructs. Supernatant containing α MHCII-HA^{PR8}

was used as a positive control in the first plate, and a biotinylated mAb specific for HA of PR8 (1:1000), (H36-4-52-bio), was used as detection antibody. Supernatant containing α NIP-HA^{Cal07} was used as a positive control in the second plate, and detection was performed with a biotinylated mAb specific for HA of Cal07 (1:1000).

2.3.2 ELISA for detection of serum antibodies

Sandwich ELISA using serum samples collected from vaccinated BALB/c mice was performed in order to measure serum antibodies against different virus HA. ELISA plates were coated with either inactivated PR8 virus (1:1600) (Charles River Laboratories), recombinant HA from Cal07 (0,5 μ g/ml) (A/california/07/2009, Sino Biological Inc), or recombinant HA from H5N1 (0,5 μ g/ml) (A/Vietnam/1194/2004, Sino Biological Inc). The protocol above was followed, except detection was performed using an antibody produced in goat, anti-mouse IgG (Fc-specific)-Alkaline phosphatase antibody (Sigma-Aldrich), that are specific for the Fc-region of mouse IgG and conjugated with alkaline phosphatase (1:5000).

2.4 ELISpot assay

For monitoring cell-mediated immunity, an Enzyme-Linked Immunospot assay can be applied due to its sensitive and accurate detection of rare antigen-specific T cells. The ELISpot assay (fig. 2.3) employs a technique very similar to the enzyme-linked immunosorbent assay (ELISA). A capture antibody with specificity for a certain analyte is coated onto a PVDF-backed microtiter plate. The plate is then blocked with serum protein that is non-reactive with any of the antibodies in the assay. Cells of interest are plated out at varying densities in the presence of varying stimuli. The proteins secreted by the cells will be captured by specific antibodies on the surface. Detection is then performed in a procedure similar to that employed by ELISA, leading to an end result where visible spots on the surface corresponds to a cytokine-producing T cell. The spots correspond to the frequency of cytokine-secreting cells at the single-cell level.

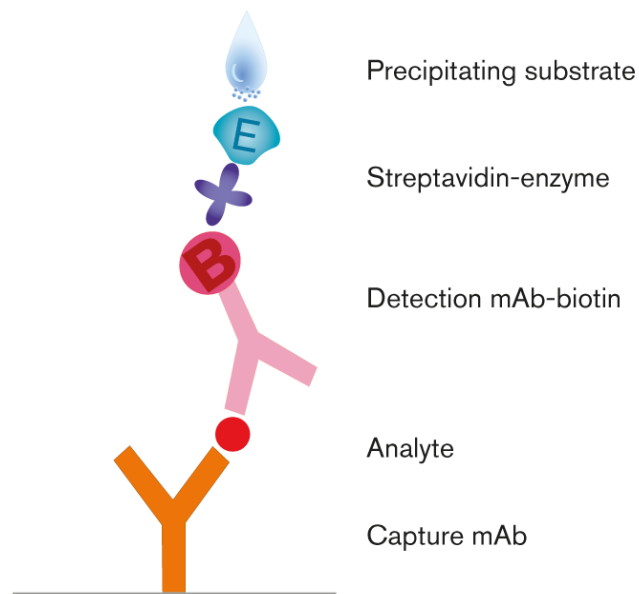


Figure 2.3. **Schematic illustration of the principle of the ELISpot assay:** Cells are cultured on a surface coated with a capture antibody specific for a certain analyte. Proteins, such as cytokines, are secreted by the cells and captured onto the surface. The secreted molecules (analyte) are detected using a biotinylated detection antibody, and followed by a streptavidin-enzyme conjugate. A precipitating substrate is added in order to create visible spots on the surface. Copyright 2015 by Mabtech[77]

In this study, plates precoated with anti-mouse IFN- γ , *Mouse IFN-gamma ELISpot PLUS kit* (ALP) (Mabtech), was applied. Blocking was performed with complete tissue culture medium (RPMI-1640 containing 10% FCS and supplements). Spleenocytes harvested from HA¹-vaccinated mice at day 28 following vaccination were added to the plates (protocol in Appendix 2). The cells were stimulated to produce IFN- γ by recHA of H1N1 A/Puerto Rico/8/34 (Sino Biological Inc), recHA of H1N1 A/California/7/2009 (Sino Biological Inc), recHA of H5N1 A/Vietnam/1194/2004 (Sino Biological Inc), and recHA of H7N9 A/Shanghai/1/2013 (Sino Biological Inc). All protein concentrations were 10 μ g/ml. As a positive control, cells were stimulated with ConA, which gives an unspecific activation of the cells. Cells without stimulation were used as negative control, in order to indicate the number of spontaneously secreting cells. Detection was performed with biotinylated anti-mouse IFN- γ and with streptavidin-horse radish peroxidase (**strep-HRP**) (GE Healthcare). The ELISpot was performed in accordance to the manufacturers protocol, and spots were counted in an Immunospot ELISpot reader (CTL).

2.5 Animal experiments

6-8 weeks old female BALB/c mice (Taconic, Denmark) were used in all animal experiments. Mice were kept at the Department of Comparative Medicine, Oslo University Hospital. Vaccination experiments were performed in the minimal disease unit (MDU), where the mice were regularly tested for pathogens to meet the Federation of European Laboratory Animal Science Association (FELASA) guidelines for health monitoring. Prior to challenge, the mice were transferred to the infectious unit (INF) at the Centre of Comparative Medicine. All animal experiments were approved by the National Committee for Animal Experiments (Oslo, Norway).

2.5.1 Intradermal vaccination

Intradermal injection is the injection of a substance directly into the dermis of the skin, just below the epidermis. For intradermal delivery of vaccine, 6 BALB/c mice per group were anaesthetized by intraperitoneal (i.p.) injection of 150 µl of ZRF and shaved in the lower back region. DNA plasmids dissolved in NaCl were injected intradermally on each flank of the mouse (a total of 25 µg DNA per mouse). Skin electroporation (EP) with DermaVax (Celectis) was performed immediately after injection, in order to increase uptake of DNA by skin cells.

2.5.2 Intramuscular vaccination

Intramuscular injection is the injection of a substance directly into a muscle. For intramuscular delivery of vaccine, 6 BALB/c mice per group were anaesthetized by i.p. injection of 150 µl of ZRF and shaved on the outer side of each hind leg. 25 µl of plasmids dissolved in NaCl were injected intramuscularly on each leg (a total of 50 µg of DNA per mouse). Skin electroporation (EP) with an ELGEN pulse generator (INOVIO Pharmaceuticals) was performed immediately after injection with needles long enough to penetrate the muscle.

2.5.3 Blood sampling

Blood samples were collected by puncture of the saphenous vein. The mouse was restrained in a plastic tube with an air hole in the end. The leg was shaved in the direction of hair growth with a scalpel blade until vein was visible. The scalpel was held as flat as possible in order to prevent cuts. The vein was then punctured with a needle held approximately 90 degrees to the skin and the blood was collected in numbered microcentrifuge tubes.

Separating serum from blood cells

After collection of blood samples, the tubes were centrifuged in a table top centrifuge at 13 000 rpm for 10 minutes. The resulting supernatant was collected and transferred into a clean microcentrifuge tube. Centrifugation was repeated and supernatant (serum) was collected into a third tube. The serum was stored at -20°C.

2.5.4 Viral challenge

Groups of vaccinated mice (n = 6/group) were anaesthetized by i.p. injection of 150 µl of ZRF prior to challenge with influenza. The mice were infected intranasally with 5xLD₅₀ of either A/Puerto Rico/8/1934 or A/H1N1/Cal/07/09 in 10 µl per nostril at 4 weeks past vaccination. Mice were monitored for weight loss with an end point of 20% weight reduction, as required by the National Committee for Animal Experiments. Mice reaching the limit of >20% weight loss were euthanized by either cervical dislocation or by use of a CO₂ chamber.

2.6 Statistical analyses

The data produced by the ELISpot was analyzed using a Mann-Whitney test. Standard deviation (SD), standard error of the mean (SEM), were calculated by GraphPad Prism 6.

3 Results

The main objectives of this thesis are: (1) to construct the mosaic hemagglutinin antigens and the modified influenza vaccine constructs, (2) to demonstrate correct folding and receptor binding for the vaccine constructs, (3) show that mice immunized with the novel vaccines can induce relevant antibodies and T cell responses.

3.1 Construction of mosaic hemagglutinin antigens

3.1.1 Phylogenetic trees of collected hemagglutinin sequences

Two mosaic antigens were generated using the Mosaic Vaccine Designer tool. One was based on hemagglutinin sequences from the H1 subtype (HA¹). The second one was based on hemagglutinin sequences from 17 subtypes of HA (HA¹⁻¹⁷). A phylogenetic tree was made for all collected H1 sequences, and the more distantly related sequences were manually selected as input sequences for insertion into the Mosaic Vaccine Designer tool. For generation of the HA¹⁻¹⁷ mosaic, phylogenetic trees were made for subtypes H2, H4, H5, H6, H7, and H9. Sequences were collected manually from each tree and used as input sequences. Figure 3.1 shows the tree built for the H5 subtype, and the other trees are shown in Appendix 4: Results.

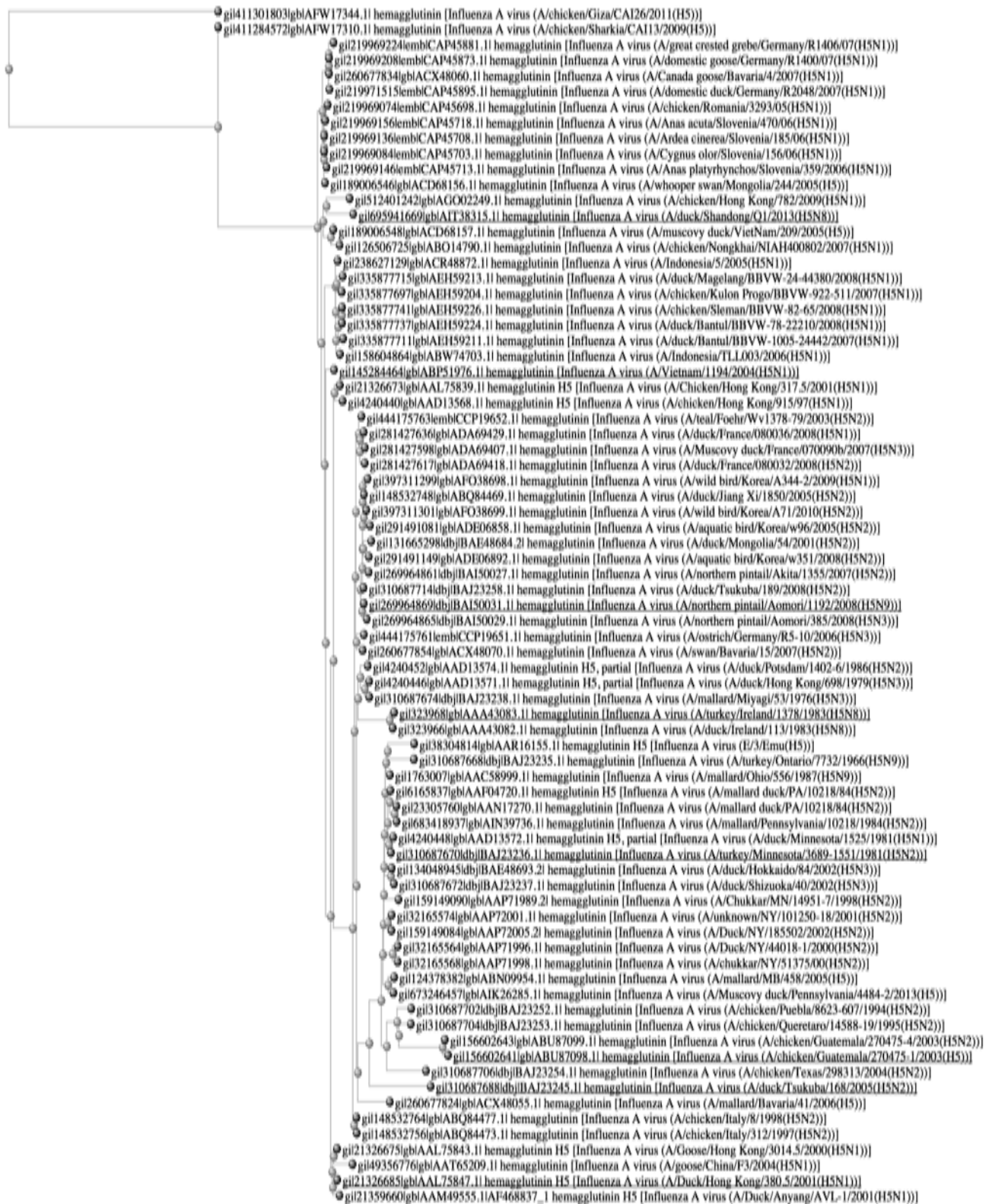


Figure 3.1. Phylogenetic tree of collected H5 sequences: Phylogenetic tree displaying the evolutionary relationships between 77 H5 sequences collected from the NCBI protein database. Underlined sequences were selected to be used as input sequences.

3.1.2 Alignments

The two mosaic antigens made with the Mosaic Vaccine Designer contains only natural T cell epitopes, but are still novel sequences. Pairwise sequence alignments were performed for both antigens against the hemagglutinin protein sequences of A/Puerto Rico/8/1934 (PR8) and A/California/07/09 (Cal07), in order to identify regions of similarity between the viral sequences and each mosaic. These viruses were later applied in viral challenge with mice vaccinated with the mosaic antigens. Regions of similarity indicate evolutionary relationships between the sequences, and also functional and structural similarities. The alignments show that differences between the aligned sequences is found predominantly in the variable region of hemagglutinin, that encodes the globular head region. Less differences are found in the conservative region, that encodes the stalk domain of HA (fig. 3.2).

A

Mosaic	1	DTICIGYHANNSTDVDTVLEKNVTVTHSVNLLLED SHNGKLC LLKGIAPLQLGKCNIAGW	60
PR8	1	DTICIGYHANNSTDVDTVLEKNVTVTHSVNLLLED SHNGKLC RLKGIAPLQLGKCNIAGW	60
Mosaic	61	LLGNPEC D L L L T A N S W S Y I I E T S N S E N G T C Y P G E F I D Y E E L R E Q L S S V S S F E R F E I F P K T	120
PR8	61	LLGNPEC D P L L P V R S W S Y I V E T P N S E N G I C Y P G D F I D Y E E L R E Q L S S V S S F E R F E I F P K E	120
Mosaic	121	SSWPNH E T T K G V T A A C S Y S G A S S F Y R N L L W I T K K G T S Y P K L S K S Y T N N K G K E V L V L W G V H	180
PR8	121	SSWPNH - N T N G V T A A C S H E G K S S F Y R N L L W L T E K E G S Y P K L K N S Y V N K K G K E V L V L W G I H	179
Mosaic	181	HPPN I G D Q R A L Y H T E N A Y V S V S S H Y S R R F T P E I A A R P K V R G Q A G R M N Y Y W T L L D Q G D T I	240
PR8	180	HPPN S K E Q Q N L Y Q N E N A Y V S V T S N Y N R R F T P E I A E R P K V R D Q A G R M N Y Y W T L L K P G D T I	239
Mosaic	241	T F E A T G N L I A P W Y A F A L S R G F G S G I I T S N A P M D E C D A K C Q T P Q G A I N S S L P F Q N V H P V T I	300
PR8	240	I F E A N G N L I A P M Y A F A L S R G F G S G I I T S N A S M H E C N T K C Q T P L G A I N S S L P Y Q N I H P V T I	299
Mosaic	301	G E C P K Y V K S T K L R M A T G L R N I P S I Q S R G L F G A I A G F I E G G W T G M I D G W Y G Y H H Q N E Q G S G	360
PR8	300	G E C P K Y V R S A K L R M V T G L R N I P S I Q S R G L F G A I A G F I E G G W T G M I D G W Y G Y H H Q N E Q G S G	359
Mosaic	361	Y A A D Q K S T Q N A I N G I T N K V N S V I E K M N T Q F T A V G K E F N K L E R R M E N L N K K V D D G F L D I W T	420
PR8	360	Y A A D Q K S T Q N A I N G I T N K V N T V I E K M N I Q F T A V G K E F N K L E K R M E N L N K K V D D G F L D I W T	419
Mosaic	421	Y N A E L L V L L E N E R T L D F H D S N V K N L Y E K V K S Q L K N N A K E I G N G C F E F Y H K C D D E C M E S V K	480
PR8	420	Y N A E L L V L L E N E R T L D F H D S N V K N L Y E K V K S Q L K N N A K E I G N G C F E F Y H K C D N E C M E S V R	479
Mosaic	481	N G T Y D Y P K Y S E E S K L N R E K I D G V K L E S M G V Y Q I L A I Y S T V A S S L	524
PR8	480	N G T Y D Y P K Y S E E S K L N R E K V D G V K L E S M G I Y Q I L A I Y S T V A S S L	523
Mosaic	1	D T I C I G Y H A N N S T D V D T V L E K N V T V T H S V N L L E D S H N G K L C L L K G I A P L Q L G K C N I A G W	60
Cal07	1	D T L C I G Y H A N N S T D V D T V L E K N V T V T H S V N L L E D K H N G K L C K L R G V A P L H L G K C N I A G W	60
Mosaic	61	L L G N P E C D L L L T A N S W S Y I I E T S N S E N G T C Y P G E F I D Y E E L R E Q L S S V S S F E R F E I F P K T	120
Cal07	61	I L G N P E C E S L S T A S S W S Y I V E T S S D N G T C Y P G D F I D Y E E L R E Q L S S V S S F E R F E I F P K T	120
Mosaic	121	SSWPNH E T T K G V T A A C S Y S G A S S F Y R N L L W I T K K G T S Y P K L S K S Y T N N K G K E V L V L W G V H	180
Cal07	121	SSWPNH D S N K G V T A A C P H A G A K S F Y K N L I W L V K K G N S Y P K L S K S Y I N D K G K E V L V L W G I H	180
Mosaic	181	H P P N I G D Q R A L Y H T E N A Y V S V S S H Y S R R F T P E I A A R P K V R G Q A G R M N Y Y W T L L D Q G D T I	240
Cal07	181	H P S T S A D Q Q S L Y Q N A D A Y V F V G S S R Y S K K F K P E I A I R P K V R D Q E G R M N Y Y W T L V E P G D K I	240
Mosaic	241	T F E A T G N L I A P W Y A F A L S R G F G S G I I T S N A P M D E C D A K C Q T P Q G A I N S S L P F Q N V H P V T I	300
Cal07	241	T F E A T G N L V V P R Y A F A M E R N A G S G I I S D T P V H D C N T T C Q T P K G A I N T S L P F Q N I H P I T I	300
Mosaic	301	G E C P K Y V K S T K L R M A T G L R N I P S I Q S R G L F G A I A G F I E G G W T G M I D G W Y G Y H H Q N E Q G S G	360
Cal07	301	G K C P K Y V K S T K L R L A T G L R N V P S I Q S R G L F G A I A G F I E G G W T G M V D G W Y G Y H H Q N E Q G S G	360
Mosaic	361	Y A A D Q K S T Q N A I N G I T N K V N S V I E K M N T Q F T A V G K E F N K L E R R M E N L N K K V D D G F L D I W T	420
Cal07	361	Y A A D L K S T Q N A I D E I T N K V N S V I E K M N T Q F T A V G K E F N H L E K R I E N L N K K V D D G F L D I W T	420
Mosaic	421	Y N A E L L V L L E N E R T L D F H D S N V K N L Y E K V K S Q L K N N A K E I G N G C F E F Y H K C D D E C M E S V K	480
Cal07	421	Y N A E L L V L L E N E R T L D Y H D S N V K N L Y E K V R S Q L K N N A K E I G N G C F E F Y H K C D N T C M E S V K	480
Mosaic	481	N G T Y D Y P K Y S E E S K L N R E K I D G V K L E S M G V Y Q I L A I Y S T V A S S L	524
Cal07	481	N G T Y D Y P K Y S E E A K L N R E I D G V K L E S T R I Y Q I L A I Y S T V A S S L	524

B

Mosaic	1	DKICLGHHAVANGTKVNTLTERGVEVVNATETVERTNVPRICS-KGKRTVDLGQCGLLGT	59
PR8	1	DTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLSDHNGKLCRLKGIAPLQLGKCNIAGW	60
Mosaic	60	LIGPPQCDQFLEF-SADLIERREGSD-VCPGKFTNEESLRQILRESGGID-----KE	111
PR8	61	LLGNPECDPLLPVRSWSYIVETPNSENGICYPGDFIDYEELREQLSSVSSFERFEIFPKE	120
Mosaic	112	SMGFTYSGIRTNGATSACRRSG-SSFYAEMKWLLSNSDNAAFPQMTKSYRNPRNKPALII	170
PR8	121	S---SWPNHNTNGVTAACSHGKSSFYRNLLWL--TEKEGSYPKLKNSYVNKKGKEVLVL	175
Mosaic	171	WGIHHSGSTTEQTKLYGSGNKLITVGSSKYQSFSPGARPPQVNGQSGRIDFHWLLLDLP	230
PR8	176	WGIHHPNPSKEQQONLYQENAYVSVVTSNYNRRFTPEIAERPQVRDQAGRMNYYWTLLKP	235
Mosaic	231	NDVTFTTFNGAFIAPDRA-SFFRGESLGVQSDVPLDSSCGGDCFHSGGTIVSSLPFQNIH	289
PR8	236	GDIIIFEANGNLIAPMYAFALSRGFGSGIITSNASMHECNTKCQTPLGAINSSLPYQNIH	295
Mosaic	290	PVTIGECPKYVKSDDLVLATGLRNVPIESRGLFGAIAAGFIEGGWTGMIDGWYGYHHQNE	349
PR8	296	PVTIGECPKYVRSRSLRMVLTGLRNIPSIQSRGLFGAIAAGFIEGGWTGMIDGWYGYHHQNE	355
Mosaic	350	QSGGYAADKESTQKAIDGITNKVNSVIEKMNTQFTAVGKEFNLEKRIENLNKKMEDGFL	409
PR8	356	QSGGYAADKSTQNAINGITNKVNTVIEKMNIQFTAVGKEFNLEKRMENLNKKVDDGFL	415
Mosaic	410	DVWTYNAELLVLLLENERTLDFHDSNVKNLYEKVKSQKLNNAKEIGNGCFEFYHKCDNECM	469
PR8	416	DIWTYNAELLVLLLENERTLDFHDSNVKNLYEKVKSQKLNNAKEIGNGCFEFYHKCDNECM	475
Mosaic	470	ESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSL	517
PR8	476	ESVRNGTYDYPKYSEESKLNREKIDGVKLESMGYQILAIYSTVASSL	523
Mosaic	1	DKICLGHHAVANGTKVNTLTERGVEVVNATETVERTNVPRICS-KGKRTVDLGQCGLLGT	59
Cal07	1	DTLCIGYHANNSTDTVDTVLEKNVTVTHSVNLLSDKHNGKLCRLKGVAPLHLGKCNIAGW	60
Mosaic	60	LIGPPQCDQFLEFSADLIERREGSD--VCPGKFTNEESLRQILRESGGIDKESMGFTY	117
Cal07	61	ILGNPECESLSTASSWSYIVETSSDNGTCYPGDFIDYEELREQLSSVSSFERFEI-FPK	119
Mosaic	118	SGIRTN-----GATSACRRSGS-SFYAEMKWLLSNSDNAAFPQMTKSYRNPRNKPALIIW	171
Cal07	120	TSSWPNHDSNKGVTAACTHAGAKSFYKNLIWLKKG--SYPKLSKSYINDKGKEVLVLW	177
Mosaic	172	GIHHSGSTTEQTKLYGSGNKLITVGSSKYQSFSPGARPPQVNGQSGRIDFHWLLLDPN	231
Cal07	178	GIHHPSTSDQSSLYQNADAYVFGSSRYSKKFKEIAIRPKVRDQEGRMNYYWTLVPEPG	237
Mosaic	232	DTVTFTFNGAFIAPDRASFRRGESLG--VQSDVPLDSSCGGDCFHSGGTIVSSLPFQNI	288
Cal07	238	DKITFEATGNLVVP-RYAFAMERNAGSGIIISDTPV-HDCNTTCQTPKGAINTSLFPQNI	295
Mosaic	289	HPVTIGECPKYVKSDDLVLATGLRNVPIESRGLFGAIAAGFIEGGWTGMIDGWYGYHHQN	348
Cal07	296	HPITIGKCPKYVKS TKLRLATGLRNVPSIQSRGLFGAIAAGFIEGGWTGMVDGWYGYHHQN	355
Mosaic	349	EQSGGYAADKESTQKAIDGITNKVNSVIEKMNTQFTAVGKEFNLEKRIENLNKKMEDGF	408
Cal07	356	EQSGGYAADLKSTQNAIDEITNKVNSVIEKMNTQFTAVGKEFNHLEKRIENLNKKVDDGF	415
Mosaic	409	LDVWTYNAELLVLLLENERTLDFHDSNVKNLYEKVKSQKLNNAKEIGNGCFEFYHKCDNEC	468
Cal07	416	LDIWTYNAELLVLLLENERTLDYHDSNVKNLYEKVRSQKLNNAKEIGNGCFEFYHKCDNTC	475
Mosaic	469	MESVKNGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSL	517
Cal07	476	MESVKNGTYDYPKYSEESKLNREKIDGVKLESTRIVYQILAIYSTVASSL	524

Figure 3.2. **Alignment of hemagglutinin sequences:** Alignments of the protein sequences of A) HA¹ against H1 (PR8) and H1 (Cal07), and B) HA¹⁻¹⁷ against H1 (PR8) and H1 (Cal07). Differing amino acids are shown in red, and the amino acids encoding the globular head domain are shown with a grey background.

3.1.3 Epitope coverage assessment

The Epicover tool was used in order to compute the coverage of epitopes in a population by the mosaic genes. Coverage is defined as the mean fraction of natural-sequence 9-mers included in the antigen, averaged over all natural sequences in the input data set. As similar epitopes may cross-react, near-matches that were off-by-1 and off-by-2 amino acids were included. The coverage of the HA¹ input set epitopes was calculated for HA¹ and HA of PR8 (fig. 3.3). Followingly, the coverage of the epitopes in the HA¹⁻¹⁷ input set was calculated for HA¹⁻¹⁷ and HA of PR8 (Appendix 4). For both input sets, the mosaic hemagglutinin antigen gave a higher coverage than HA of PR8.

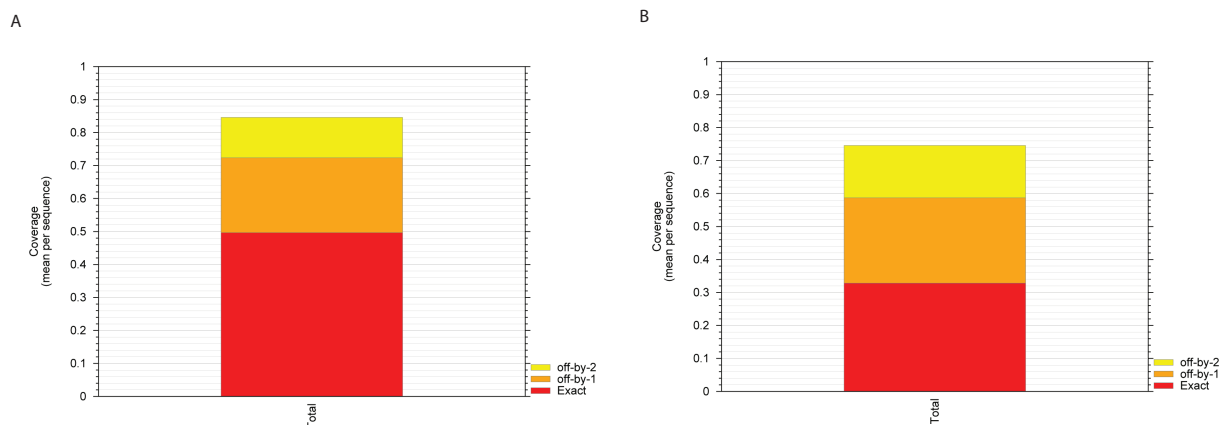


Figure 3.3. **Epitope coverage of HA¹ and H1 (PR8):** Calculation of coverage for two influenza antigens: A) The mean fraction of natural-sequence 9-mers shared with HA¹ averaged over all sequences in the HA¹ input set, and B) The fraction of 9-mers shared with H1 (PR8) averaged over all sequences in the HA¹ input set. Both exact matches (red), off-by-one matches (orange) and off-by-2 matches (yellow) were included in the score.

3.2 Characterization of vaccine constructs

We have subcloned the HA¹ and HA¹⁻¹⁷ mosaics on SfiI-sites into pLNOH2 vectors containing a previously described vaccine format that was designed to target antigens to various receptors on APCs[51, 53]. The mosaic was here equipped with four different targeting units; MIP1 α [54], XCL1[78], α MHCII[51] and α NIP[51]. MIP1 α and XCL1 are chemokines that target the chemokine receptors CCR1/3/5 and Xcr1, respectively. An MHC class II specific scFv was used for targeting to MHC class II molecules, and a scFv against the hapten NIP was utilized as a non-targeted control. The eight resulting vaccine constructs are listed in table 1.

Table 1: List of the mosaic vaccine constructs.

	Mosaic HA¹	Mosaic HA¹⁻¹⁷
αNIP	α NIP-HA ¹	α NIP-HA ¹⁻¹⁷
MIP-1α	MIP-1 α -HA ¹	MIP-1 α -HA ¹⁻¹⁷
XCL1	XCL1-HA ¹	XCL1-HA ¹⁻¹⁷
αMHCII	α MHCII-HA ¹	α MHCII-HA ¹⁻¹⁷

Supernatants of 293E cells transiently transfected with the eight different vaccine plasmids, were tested using different sandwich ELISA assays. The ELISAs were performed according to the protocol in the methods chapter (section 2.3).

An ELISA assay was performed with coating and detecting antibodies against the dimerization unit, in order to assess the secretion efficacy of the constructed vaccines. The measured OD levels were plotted against the dilution factor (fig.3.4A). The result demonstrated efficient secretion of all eight constructs *in vitro*, although differing OD values indicated that some vaccine proteins were secreted more readily than others. The α MHCII-HA¹, XCL1-HA¹⁻¹⁷, and MIP1 α -HA¹⁻¹⁷ were all expressed better than the positive control plasmid, and the NIP-targeted constructs were expressed more poorly than the others. The positive control (α MHCII-HA^{PR8}), previously shown to be secreted by 293E cells[79], confirmed that the ELISA was working properly, and the low value for mock indicates that there were no false-positive results and little non-specific binding. Other ELISA assays were performed in order to verify insertion of the α NIP - and MIP1 α targeting units into the vaccine proteins. The high OD values measured for the α NIP-HA¹, α NIP-HA¹⁻¹⁷ (fig. 3.4B), MIP1 α -HA¹, and MIP1 α -HA¹⁻¹⁷(fig. 3.4C) constructs, compared to the other samples, verified the presence of the correct targeting unit. A high OD value for the positive controls (MIP^{Pos} and NIP^{Pos}) confirmed that the ELISA was working properly for both plates, and low OD values for mock indicated that the targeting units were not falsely detected. We concluded from these results that all vaccine constructs were able to be secreted *in vitro*, and that the MIP1 α - and α NIP-targeted vaccines contained the correct targeting units. As the NIP-targeted constructs were able to bind NIP, we assume a correct folding of the α NIP-constructs.

In order to examine whether antibodies against HA of the H1 subtype could bind the mosaic antigens, ELISAs were set up with mAb against the dimerization unit as coat, and with mAb against HA from PR8 and Cal07 for detection. The modified vaccine constructs could not be recognized by the mAbs specific for H1 HAs (fig. 3.5). The high OD levels detected for the positive controls indicate that the ELISA had been functional. The lack of response from the negative control (mock) indicated that there was little non-specific binding. We concluded from this result that the modified hemagglutinin antigens have different binding capacities than the HAs of PR8 and Cal07.

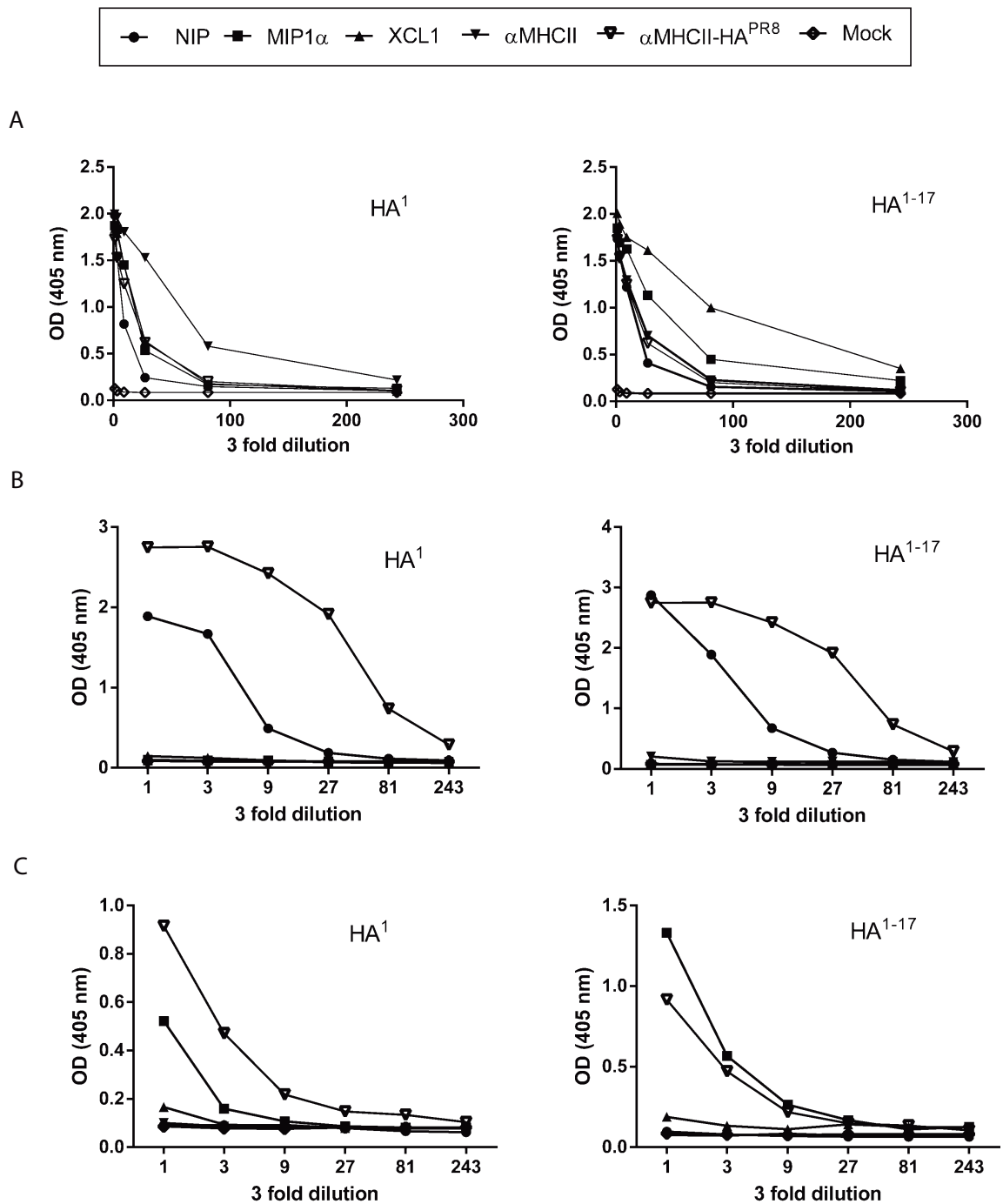


Figure 3.4. **Detection of vaccine proteins secreted *in vitro*:** ELISAs performed on dilutions of supernatants from transiently transfected 293E cells. A) ELISA plates were coated with the capture antibody MCA878 and detection was performed using HP6017-bio, both antibodies binding the dimerization unit of the vaccibodies. B) ELISA plate was coated with α NIP and detected with HP6017-bio. C) ELISA plate was coated with MIP1 α and detected with HP6017-bio.

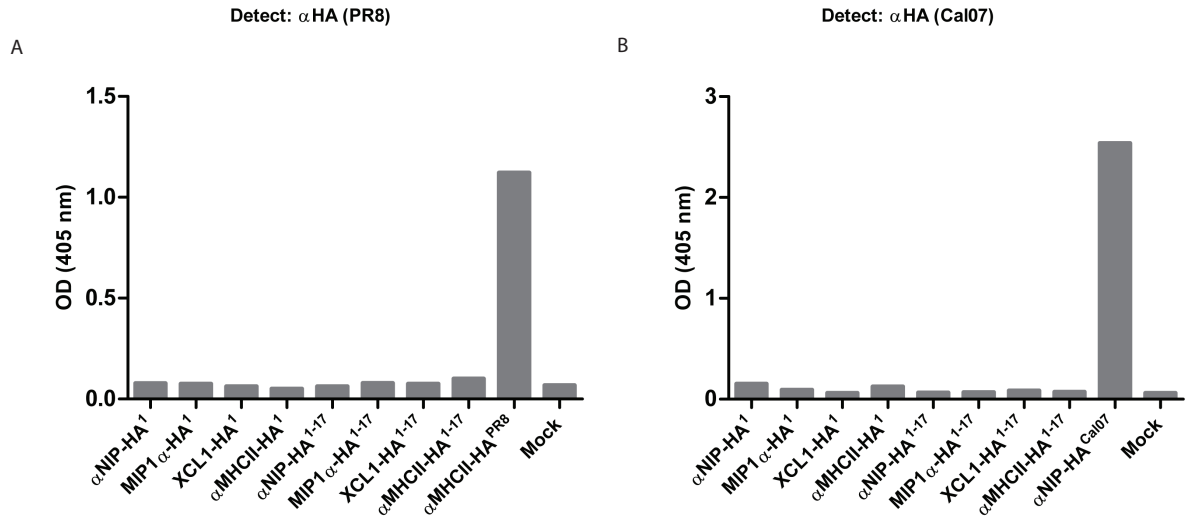


Figure 3.5. **Secreted vaccine constructs were not detected with antibodies specific for H1 HA:** Sandwich ELISAs performed on dilutions of supernatant from transient transfection of 293E cells. Plates were coated with MCA878, and detected with mAb against A) PR8, and B) Cal07.

3.3 Vaccination with mosaic vaccine constructs induces humoral responses in mice

While the mosaic HA could not be bound by mAbs against H1 HAs, it is conceivable that the mosaic antigens could nevertheless induce antibodies specific for various HAs *in vivo*. Thus, ELISAs using serum samples from mice vaccinated with the novel vaccine constructs were performed in order to measure serum antibodies against HA of a variety of viruses. A positive control group was vaccinated with the MCHII-HA^{PR8} plasmid, and a negative control group was vaccinated with NaCl. αMCHII-HA^{PR8} was used as positive control since it has previously shown to induce complete protection against a lethal dose of PR8[71, 79].

3.3.1 Vaccination with HA¹ enhances antibody titers following intradermal and intramuscular delivery

ELISAs were performed with sera from mice vaccinated with the HA¹ mosaic in order to measure the ability of the HA¹ vaccines to induce antibodies. 36 BALB/c mice (n = 6/group) were injected once intradermally with either MIP1-α-HA¹, XCL1-HA¹, αMHCII-HA¹, αNIP-HA¹, or αMHCII-HA^{PR8} plasmids, along with NaCl as a negative control. Another 36 BALB/c

mice (n = 6/group) were injected once intramuscularly with the same plasmids and NaCl. The vaccinations were performed as described in the methods chapter (section 2.5.1 and 2.5.2).

In order to investigate induction of PR8-specific antibodies, antibody titers were measured for sera collected 1, 2, and 4 weeks following a single vaccination (fig. 3.6). This ELISA assay showed that there was a slight increase in sera of PR8-specific antibodies during these four weeks for mice vaccinated with MIP1 α -HA¹, XCL1-HA¹, and α NIP-HA¹, both intradermally and intramuscularly. The α MCHII-HA^{PR8} largely enhanced large antibody titers against PR8 for both delivery strategies. The intramuscular vaccination gave higher titers than the intradermal vaccination with α MCHII-HA^{PR8}, and slightly higher titers with MIP1 α -HA¹, XCL1-HA¹, and α NIP-HA¹. α MCHII-HA¹ failed to induce antibodies against PR8 in both experiments.

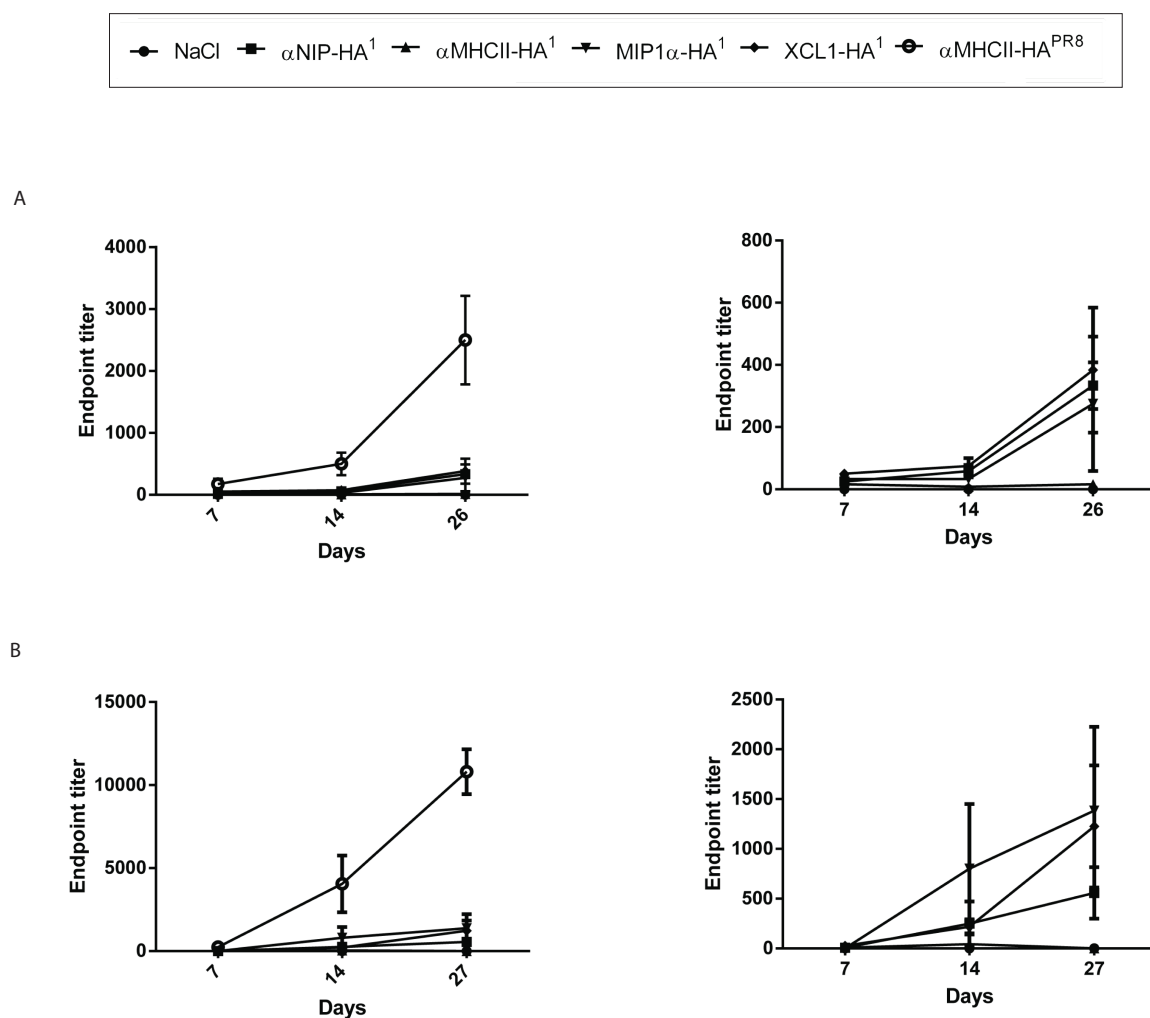


Figure 3.6. **Increase in PR8-specific titers following intradermal and intramuscular vaccination with HA¹:** Microtiter plates were coated with PR8 and detection was performed with anti-mouse IgG

in order to investigate induction of PR8-specific antibodies following i.d. and i.m. delivery of HA¹. Titers were measured for all constructs at week 1, 2, and 4 for intradermally (A) and intramuscularly (B) vaccinated mice. Titers were given as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five-times SEM). The right figures show only the mosaic vaccine constructs and NaCl.

In order to examine antibody responses against HAs of relevant influenza strains, ELISA plates were coated with either inactivated PR8 virus, recombinant HA of Cal07 or recombinant HA of H5N1. Serum samples from week 4 after vaccination were analyzed for HA-specific IgG. The positive control plasmid, α MHCII-HA^{PR8}, induced high titers against PR8, and thus confirmed functional vaccination. Similar to the responses measured above (fig. 3.6), the other vaccine constructs could only induce minute responses against PR8. Nevertheless, there seemed to be an increased effect after targeting of mosaic HA to chemokine receptors after i.m. vaccination.

When examining antibody responses against Cal07, the positive control, α MHCII-HA^{PR8}, failed to induce significant antibody titers. Interestingly, targeting of mosaic HA to chemokine receptors increased antibody responses, with XCL1-HA¹ being particularly noteworthy after i.d. vaccination, and MIP1 α -HA¹ after i.m. vaccination. The increased effect of MIP1 α -HA¹ also held true for measurements of antibodies against HA from H5 influenza. The intramuscular vaccination gave overall higher titers against H1 (Cal07) and H5 (H5N1), than the intradermal vaccination (fig. 3.7B). In sum, targeting of mosaic HA with MIP1 α seems particularly efficient, but XCL1-HA¹ and α NIP-HA¹ can also raise cross-reactive antibodies.

For further assessment of antibody responses after i.m. vaccination, ELISAs against recHA (Cal07) and recHA (H5N1) was performed in sera collected on days 7, 14 and 27 after vaccination. Interestingly, MIP1 α -HA¹ demonstrated increased responses against both HA from H5 and Cal07, as compared to all other vaccines. However, a closer examination showed that at day 14, XCL1-HA¹ induced the highest antibody titers (fig. 3.8A and B). The results will be further discussed in the last chapter (section 4.2.3).

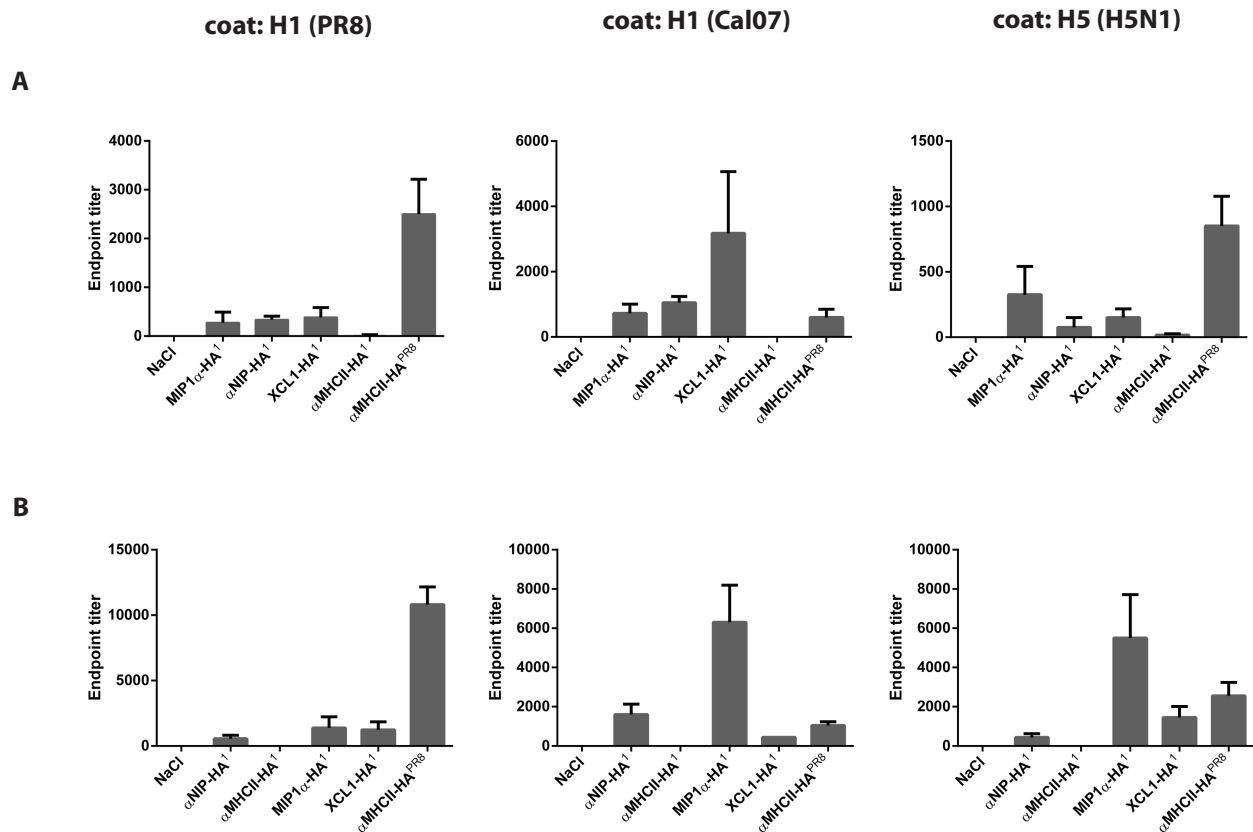


Figure 3.7. Induction of cross-protective antibodies following intradermal and intramuscular delivery of HA¹: Microtiter plates were coated with PR8, recHA (Cal07), and recHA (H5N1) and sera collected 4 weeks past vaccination were added from mice vaccinated i.d. with HA¹ (A), or from mice vaccinated i.m. with HA¹ (B). Detection was performed with anti-mouse IgG. Titers are given as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five-times SEM).

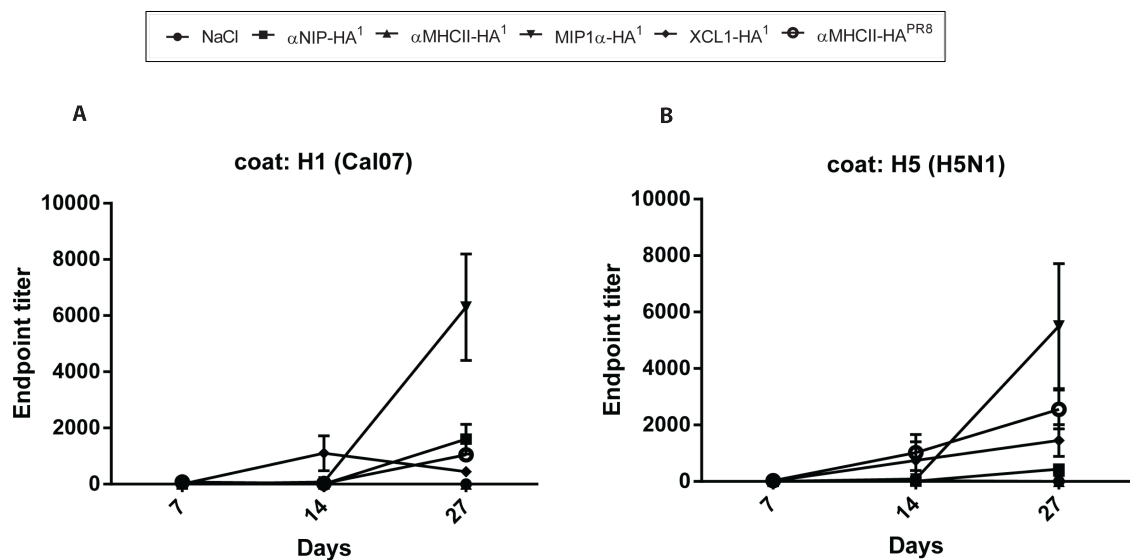


Figure 3.8. Intramuscular delivery of HA¹ induce titers against Cal07 and H5N1:

Microtiter plates were coated with recHA (Cal07) (A) or recHA (H5N1) (B) and sera collected from mice vaccinated i.m. with HA¹ at week 1, 2, and 4 were added. Detection was performed with anti-mouse IgG. Titers are given as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five-times SEM).

3.3.2 Vaccination with HA¹⁻¹⁷ enhances antibody titers following intradermal delivery

ELISAs were performed with sera from mice vaccinated with the HA¹⁻¹⁷ mosaic in order to measure the ability of the HA¹⁻¹⁷ vaccines to induce antibodies. 36 BALB/c mice (n = 6/group) were injected once intradermally with the MIP1 α -HA¹⁻¹⁷, XCL1-HA¹⁻¹⁷, α MHCII-HA¹⁻¹⁷, α NIP-HA¹⁻¹⁷, and α MHCII-HA^{PR8} plasmids, along with NaCl as a negative control, as described in the methods chapter (section 2.5.1). After the initial immunization, a booster injection was given at day 30 for re-exposure to the immunizing HA antigen, in an attempt to increase antibody responses.

ELISA plates were coated with either inactivated PR8 virus, recombinant HA of Cal07 or recombinant HA of H5N1, in order to investigate induction of cross-reactive antibodies during the 8 weeks following vaccination. The first immunization induced only low titers against all three HAs, but the second immunization boosted antibody responses (fig. 3.9.). However, it does not appear to be significant differences between the groups. Of note, vaccination with α MHCII-HA¹⁻¹⁷ did in this experiment induce antibody titers comparable to that of the other APC-targeted groups. This is in contrast to the complete lack of induced immune responses that were observed above with α MHCII-HA¹.

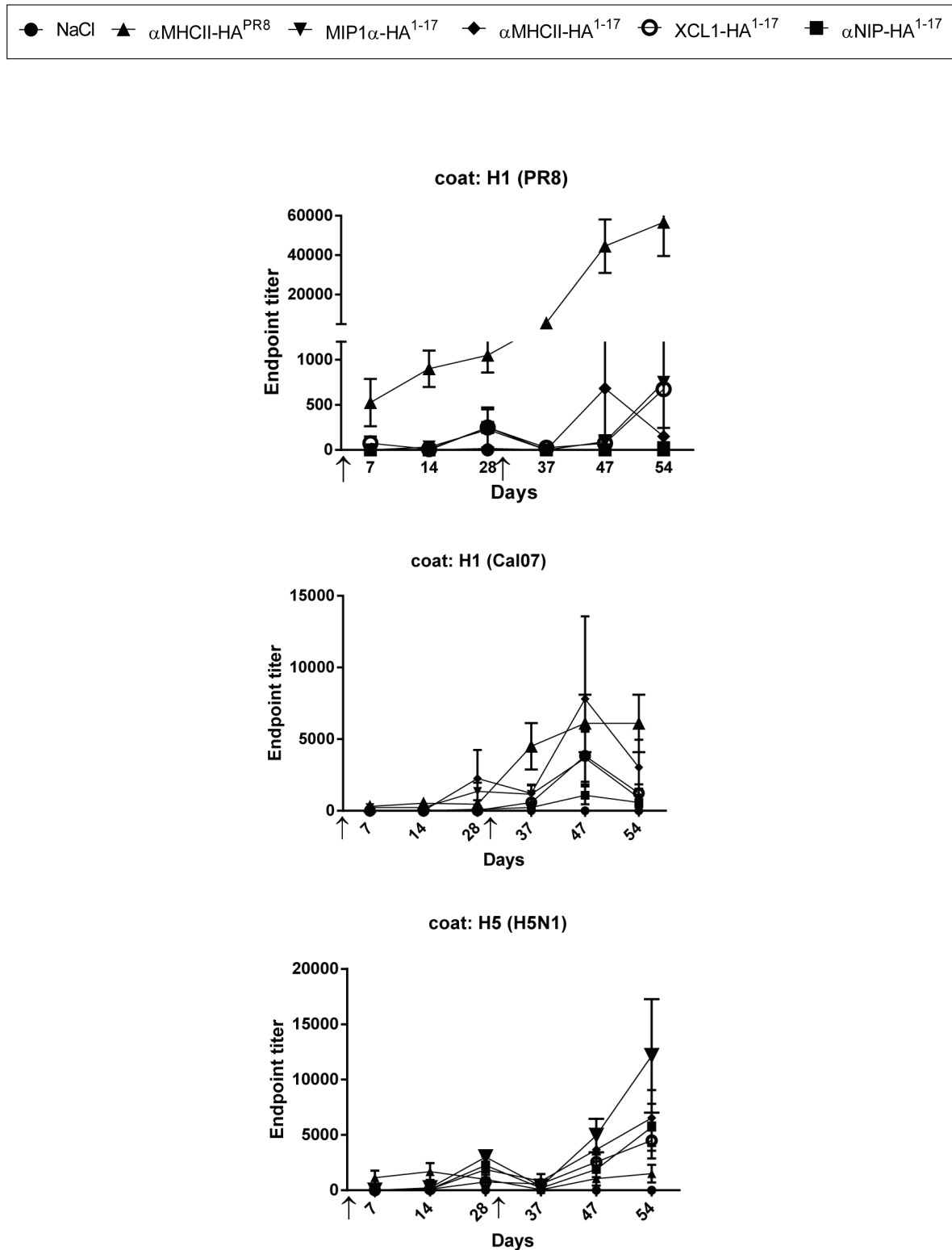


Figure 3.9: **Increase in antibody titers following intradermal vaccination with HA¹⁻¹⁷**: Mice immunized i.d. with HA¹⁻¹⁷ were assayed for total IgG against A) inactivated PR8, B) recHA (Cal07), and B) recHA (H5N1) in order to investigate induction of antibodies. Titers were measured for all constructs at week 1, 2, 4, 5, 7, and 8 and given as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five-times SEM).

The HA¹⁻¹⁷ vaccines were able to induce only low titers against PR8, but higher titers against H5 (H5N1) and H1 (Cal07). For a closer examination of the observed responses, we re-examined the measurements from weeks 4 and 8 after vaccination (fig. 3.10). The effect of APC-targeting is somewhat varied. In the ELISA with a coat of PR8, α NIP-HA¹⁻¹⁷, XCL1-HA¹⁻¹⁷ and MIP1 α -HA¹⁻¹⁷ all induces similar antibody levels at week 4 post-vaccination. However, after the boost at week 8, the responses of XCL1-HA¹⁻¹⁷ and MIP1 α -HA¹⁻¹⁷ are markedly increased above that of the non-targeted control. In the ELISA with a coat of Cal07, there is a large difference between the APC-targeted vaccines and the non-targeted control (α NIP-HA¹⁻¹⁷). At week 4, the responses after XCL1-HA¹⁻¹⁷ has not yet emerged, but these are well developed after the second vaccination in week 8. Interestingly, in this assay, it appears that α MHCII-HA¹⁻¹⁷ induces the highest levels of antibodies, but MIP1 α -HA¹⁻¹⁷ and XCL1-HA¹⁻¹⁷ are also markedly present. A targeting effect may also be observed for the H5 (H5N1) assay, as the non-targeted control group induced lower antibodies than all targeted vaccine proteins, except for XCL1-HA¹⁻¹⁷, which gave similar titers.

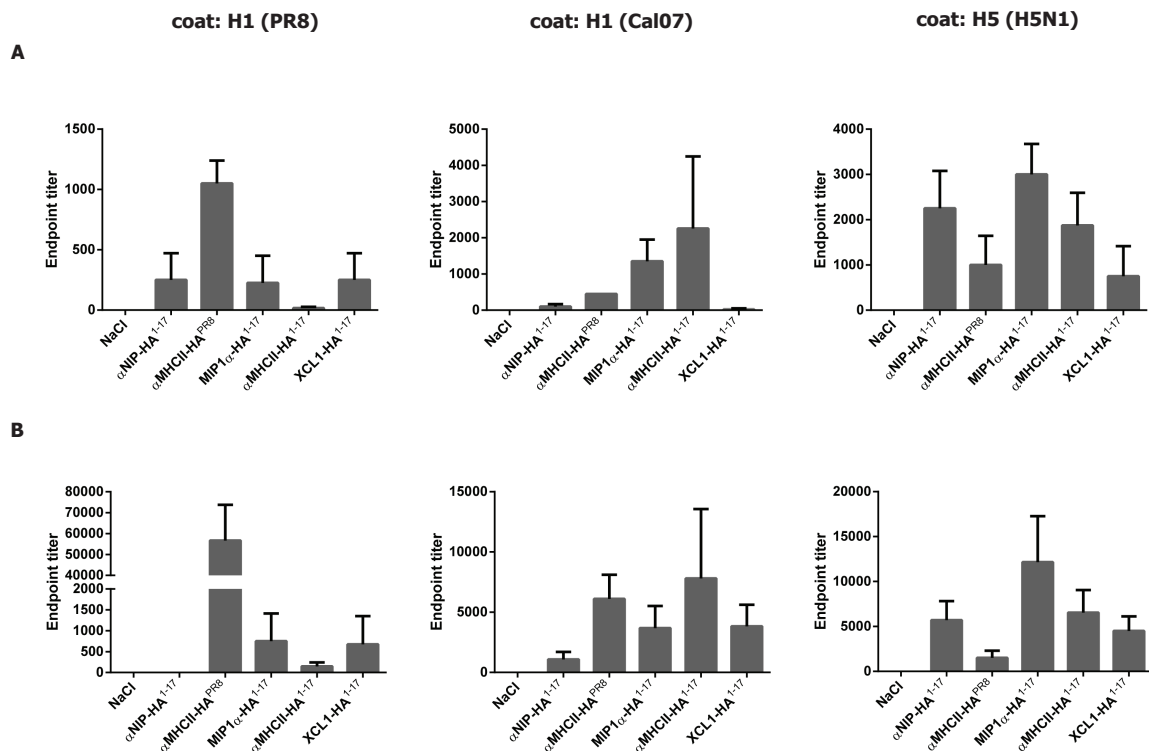


Figure 3.10. Induction of cross-protective antibodies following intradermal delivery of HA¹⁻¹⁷: Microtiter plates were coated with inactivated PR8, recHA (Cal07), or recHA (H5N1) and added sera from mice vaccinated i.d. with HA¹⁻¹⁷ in order to detect induction of cross-protective antibodies. Titers were measured at week, 4 when the mice had only received the 1. vaccination dose (A), and at week 8 (4 weeks after the 2. vaccination dose) (B). Detection was performed with anti-mouse IgG. Titers are

given as the last serum dilution giving an absorbance above background (mean absorbance for NaCl-vaccinated mice plus five-times SEM).

3.4 Targeted delivery of HA¹ with MIP1 α and XCL1 increase T cell responses

From the above ELISAs, MIP1 α -HA¹⁻¹⁷ and XCL1-HA¹⁻¹⁷ appear to be the more promising vaccines that encodes mosaic HA. Thus, an ELISpot assay was performed in order to quantify the ability of MIP1 α -HA¹, XCL1-HA¹, and α NIP-HA¹ to activate IFN γ -secreting cells (fig. 3.11). 24 mice (n=6/group) were injected once with 25 μ g DNA/EP as indicated in the methods chapter (section 2.5.1), and the ELISpot was performed according to the protocol in the methods chapter (section 2.4). Mouse spleens were harvested 4 weeks after i.d. vaccination with MIP-1 α -HA¹, XCL1-HA¹, α NIP-HA¹, and NaCl (protocol in Appendix 2). Cells were stimulated *in vitro* with recombinant HA proteins from PR8, Cal07, H5N1, and H7N9, as well as medium alone and ConA as positive control. The number of spots in the wells stimulated with ConA was too numerous to count for all groups, and so the results were set to a default of 500 spots/well (this is in accordance with the protocol developed for the ELISpot reader). A further examination showed that targeting with XCL1 activated the highest number of IFN γ -secreting cells for all stimulants, including stimulation with HAs from PR8, Cal07, H5N1, H7N9, and medium alone. However, responses after stimulation with HA from PR8 and Cal07 were higher than the other, indicating that these could represent reliable data. The number of activated IFN γ -expressing cells with XCL1 was not significantly higher for the PR8- or Cal07-stimulated cells, than the negative control cells.

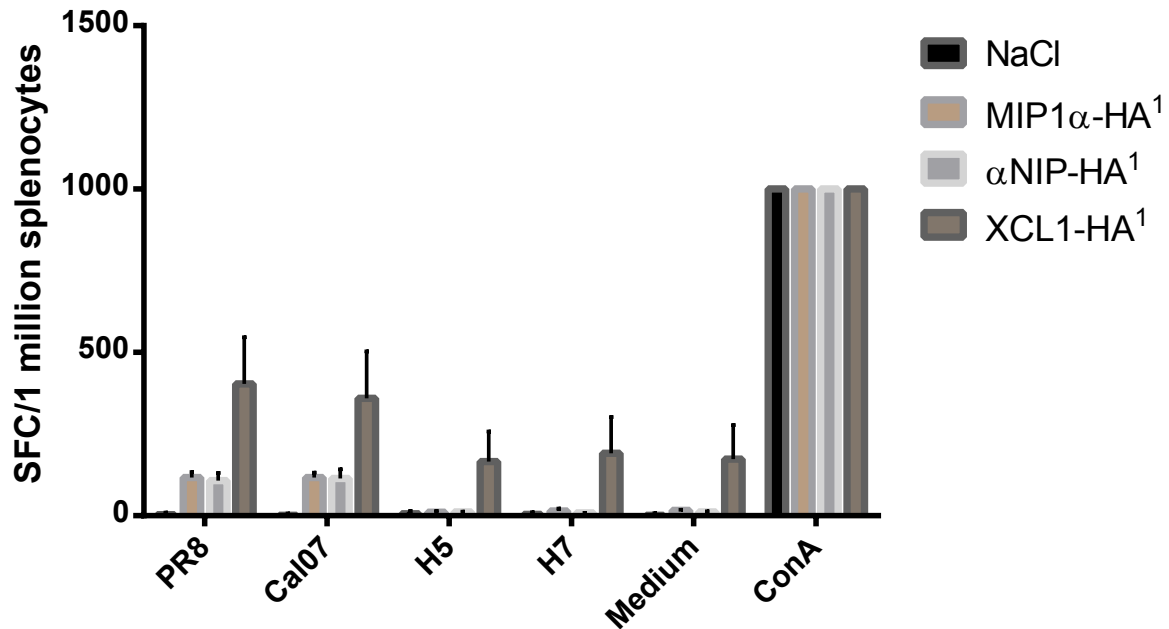


Figure 3.11. **Activation of IFN γ -secreting cells following vaccination with MIP1 α -HA¹, XCL1-HA¹, and α NIP-HA¹:** ELISpot measuring IFN γ -secreting cells was performed on splenocytes collected 4 weeks after i.d. vaccination. Splenocytes were stimulated with recombinant HA from PR8, Cal07, H5N1 and H7N9 as indicated.

3.5 Mosaic vaccine constructs provide protection against viral challenge with influenza

The ultimate testing of vaccine efficacy, is to examine vaccine-conferred protection against influenza. Thus, influenza challenge was performed with a lethal dose of either A/Puerto Rico/8/1934 or A/H1N1/California/07/09, as described in the methods chapter (section 2.5.4).

3.5.1 Viral challenge in mice following intradermal delivery of HA¹

Mice that had received i.d. injection of the various plasmids encoding HA¹ were challenged with a lethal dose of PR8 4 weeks after vaccination. Weight loss and survival proportions for each vaccine construct during the next 11 days is shown in figure 3.12. Results show that mice receiving saline or α MHCII-HA¹ had to be euthanized by day 8. Mice vaccinated with α MHCII-HA^{PR8} (positive control) showed only a minor reduction in weight before restoring their initial start weight. Mice vaccinated with the remaining constructs, α NIP-HA¹, MIP1 α -

HA¹ and XCL1-HA¹, showed a similar weight loss for the first 6 days. The αNIP-HA¹- and MIP1α-HA¹-vaccinated mice then gained weight until about 95% of total body weight was restored. The XCL1-HA¹-vaccinated mice had a slightly faster weight gain and restored their weight 100% by day 11. The positive control group was the only group where all mice survived. The survival proportions were higher for the MIP1α-HA¹-vaccinated mice (85%) than for mice vaccinated with XCL1-HA¹ (50%) and αNIP-HA¹ (50%) (fig. 3.12). The survival proportions of αMHCII-HA¹-vaccinated mice were similar to the negative control group.

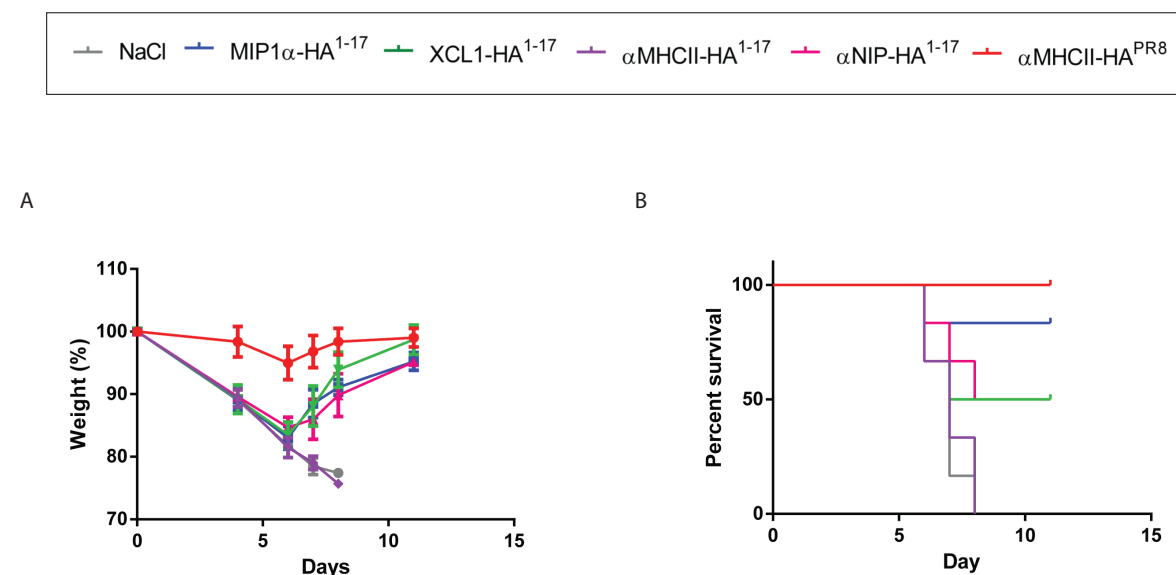


Figure 3.12. **Weight loss and survival proportions of mice vaccinated i.d. with HA¹-encoding vaccine constructs:** 36 mice vaccinated i.d. with HA¹ was monitored during the 11 days following lethal challenge with PR8 for A) weight loss, and B) survival. Mice were euthanized when reaching the limit of >20% weight loss.

3.5.2 Viral challenge in mice following intramuscular delivery of HA¹

Mice that had received i.m. vaccination with the various HA¹-constructs were challenged with a lethal dose of Cal07 4 weeks after vaccination. Weight loss and survival proportions for each HA¹ vaccine construct during the next 11 days is shown in figure 3.13. Mice that received saline had to be euthanized by day 8. The MIP1α-, XCL1-, and αNIP-targeted vaccines gave similar weight curves that eventually restored the initial weight of all mice in each group. Mice vaccinated with αMHCII-HA^{PR8} (positive control) lost more weight than the

mosaic vaccine constructs, except for α MHCII-HA¹. All mice vaccinated with either MIP1 α -HA¹, XCL1-HA¹, α NIP-HA¹, or α MHCII-HA^{PR8} survived until the experiment was terminated at day 12. Only 1 of the mice vaccinated with α MHCII-HA¹ survived until day 12.

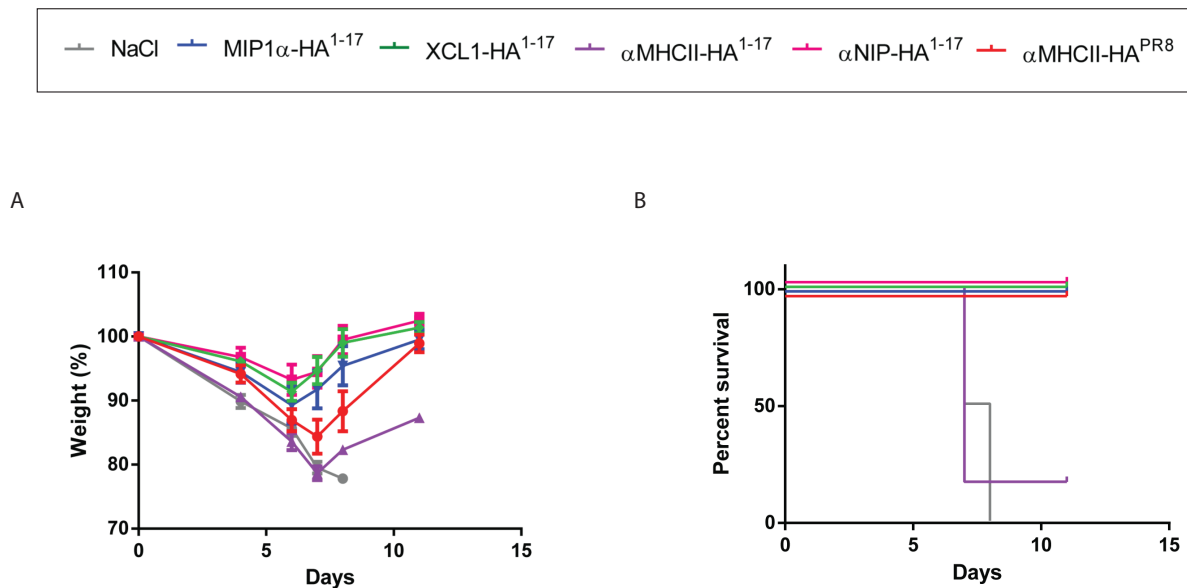
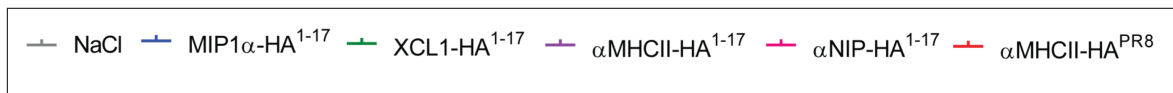


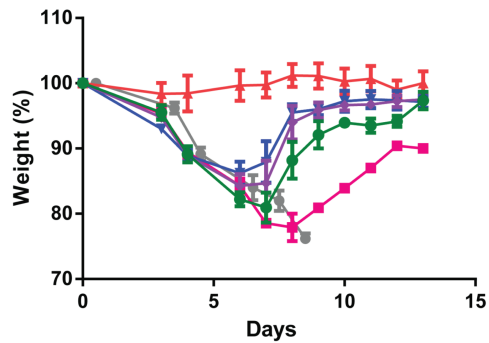
Figure 3.13. **Weight loss and survival proportions of mice vaccinated i.m. with HA¹-encoding vaccine constructs:** 36 mice vaccinated i.m. with the various plasmids encoding HA¹ was monitored during the 11 days after lethal challenge with Cal07 for A) weight loss, and B) survival. Mice were euthanized when reaching the limit of >20% weight loss.

3.5.3 Viral challenge in mice following intradermal delivery of HA¹⁻¹⁷

Mice that had received i.d. vaccination with the HA¹⁻¹⁷ encoding vaccine constructs were challenged with a lethal dose of PR8 8 weeks after vaccination. The mice had then been vaccinated twice, and were challenged 4 weeks after the second dose. Weight loss and survival proportions for each HA¹⁻¹⁷ vaccine construct during the next 13 days is shown in figure 3.14. Mice that received saline had to be euthanized by day 8. Mice vaccinated with α MHCII-HA^{PR8} were completely protected against the lethal dose of PR8. MIP1 α -HA¹⁻¹⁷, XCL1-HA¹⁻¹⁷ and α MHCII-HA¹⁻¹⁷ all had comparable weight curves. The weight curve of the α NIP-HA¹-vaccinated mice shows a more rapid weight loss than with any other group, and only one of them survived until day 13. All mice in the positive control group survived, in consistence with previous experiments with this vaccine construct (Grødeland 2013). Of the remaining groups, the MIP1 α -targeting induced the highest survival rate, followed by targeting with α MHCII, and then XCL1.



A



B

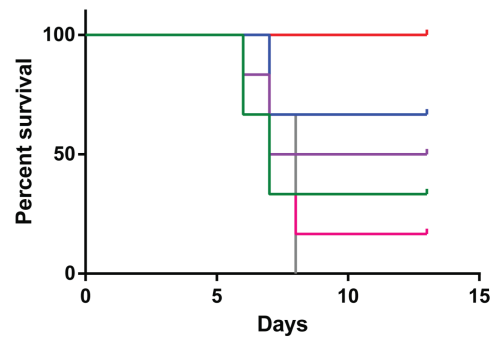


Figure 3.14: **Weight loss and survival proportions of mice vaccinated i.d. with HA¹⁻¹⁷-encoding vaccine constructs:** 36 mice were injected twice (week 0 and 4) i.d. with the indicated vaccine constructs encoding HA¹⁻¹⁷, followed by a lethal challenge with influenza PR8 at week 8. The mice were then monitored for 13 days after the challenge with PR8 for A) weight loss, and B) survival. Mice were euthanized when reaching the limit of >20% weight loss.

4 Discussion

As presented in aims of study in section 1.4, there is a need for novel influenza vaccines that can confer a broader protection against influenza. In this study we have constructed novel mosaic hemagglutinin genes that were used as antigenic unit in APC-targeted vaccine proteins, delivered as DNA. We demonstrated efficient secretion of the constructs, and examined vaccine induced immune responses in mice. Importantly, mice immunized with mosaic HA were protected against a lethal challenge with influenza. This chapter will discuss the findings and methods applied in this work, and lastly the future prospects.

4.1 Methodological considerations

4.1.1 Generation of mosaic antigens

The parameters in the Mosaic Vaccine Designer tool can be set to a wide variety of settings and in that way generate a wide variety of mosaics from the same input set. We decided in this study to generate 1 peptide that represented all uploaded sequences. Previous studies employing the mosaic vaccine designer has shown that coverage (the mean fraction of natural-sequence k -mers included in the antigen, averaged over the set of natural sequences) is increased with increasing numbers of peptides in the antigen cocktail. One example is the generation of a mosaic cocktail for use as antigen in a novel vaccine against HIV[69]. Despite demonstrating a higher coverage using more peptides, the study also showed an increased coverage compared to a natural sequence, a conserved sequence, and a consensus sequence, when applying a single mosaic peptide[66].

The aim of the present project was to generate a novel vaccine efficient at induction of cellular responses. The basis when epitope length was chosen, is that epitopes of CD8 T cells are continuous and typically 9 amino acids long. The mosaics generated in previous studies with HIV also utilized 9-mers[66, 69]. We therefore generated and scored mosaics based on the coverage of stretches of 9 contiguous amino acids in an attempt to stimulate cytotoxic T cells. Peptides binding MHC class II molecules, that are presented to CD4 T cells, display a great variability in length, ranging from 9-22 aa. Therefore, epitopes included in the mosaics

might in some instances also match the length of natural CD4 T cell epitopes. In a recent attempt to create a broader protective vaccine against influenza, an H5 mosaic was generated, setting the epitope length to 12 aa[2]. Here, they aimed at capturing CD4 epitopes, and induce antibodies that could cross-react within H5 strains of influenza. The vaccine generated showed an ability to confer broad protection against heterologous H5 influenza strains.

A higher amount of input sequences will on a general basis lead to a better coverage of T cell epitopes. Still, the number of input sequences for generation of the HA¹ mosaic was only 18. We ended up with this number after several attempts with larger input sets that all resulted in generation of a sequence with similar counterparts in nature. We reasoned that this could be due to an uneven representation of particular clusters, where the high degree of almost identical sequences present in the input would have an undue influence on the generated mosaic sequence. Thus, we constructed a tree, and selected evolutionary diverse strains as input.

For construction of the HA¹⁻¹⁷ mosaic, we used the same approach as for HA¹. For several subtypes of influenza, such as H1 and H5, there are substantially more sequences available, as compared with other subtypes. The inclusion of all available sequences would therefore have biased the mosaic HA in favour of these well-characterized subtypes at the expense of others. Thus, we made evolutionary trees of well-described subtypes, and selected the more evolutionary diverse sequences as input sequences. For subtypes where less sequences are available, all were used as input sequences.

Due to the low number of sequences, the rare threshold was set to 1, meaning every 9-mer present in the set of input sequences counts in the score. This was done because it was impossible for the algorithm to generate recombinant sequences with such a small input set without including the rare 9-mers. We tried using a higher rare threshold, but did not manage to get a resulting sequence from these attempts. A higher threshold might have lead to a more optimal mosaic, as the rarest 9-mers would not have been counted in the score. Although, in this particular case, a low rare threshold might not have meant that much, as the sequences in the input set are very diverse. Alternatively, we could have made a mosaic that was based on fewer subgroups, in order to have a larger input set and still an even distribution of the subgroups.

4.1.2 Cloning of mosaic HA encoding vaccine molecules

The pLNOH2 vector was used as expression vector due to its ability to provide efficient uptake and secretion of Vaccibodies encoding different targeting units as well as antigens from different viruses and cancer models[51, 80]. It was originally designed for efficient production of Ig heavy chains, and therefore contains a V_H leader sequence, followed by two cassettes for cloning of V_H and C_H regions. The pLNOH2 vector has a CMV promoter upstream of the V cassette, where the targeting units are cloned in. The antigenic units are cloned into the C cassette together with the dimerization unit (fig. 4.1). In this study, two mosaic HA sequences were both inserted into four different Vaccibody plasmids, each plasmid containing a specific targeting unit. The vaccine plasmids were transiently transfected into 293E cells, and ELISA analysis of supernatants demonstrated secretion of the vaccibody proteins.

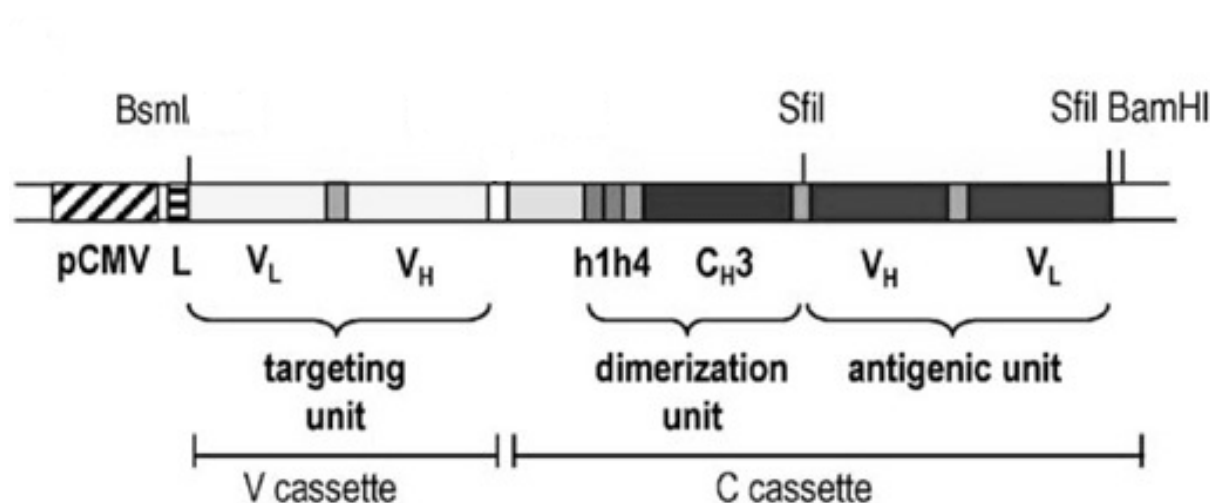


Figure 4.1. **Gene construct encoding a vaccibody:** The targeting unit (MIP1 α , XCL1, α MHCII or α NIP) is inserted into the V cassette of the pLNOH2 vector. An antigenic unit (mosaic gene) is inserted into the C cassette, next to the dimerization unit, which is composed of a shortened Ig hinge (h1 and h4 hinge exons) and a C_H3 domain of human IgG[81]. Figure adapted from Fredriksen (Fredriksen 2006).

4.1.3 Detection of antibodies in sera

Inactivated influenza PR8, recombinant H1 from Cal07, and recombinant H5 from H5N1 was used as coat in the enzyme-linked immunosorbent assay (ELISA) for measurement of antibodies against these different HAs. The vaccine-encoded mosaic HA should be the only influenza antigen that the vaccinated mice could have been exposed to. The negative control

groups, vaccinated with NaCl, remained negative for serum antibodies specific for any of the HAs throughout the experiments. Although ELISA measurements give quantitative information about antigen-specific antibodies in sera, they do not give information about these antibodies' ability to confer protection against influenza. In order to test the neutralizing ability of the antibodies, a hemagglutinin-inhibition assay (HI-assay), or a neutralization assay, could have been performed. However, non-neutralizing antibodies can also provide protection against influenza by activating complement or by binding to Fc receptors on macrophages and NK cells. These antibodies may not be recognized by either HI-assays or neutralization assays, but will be readily detected in ELISAs.

4.1.4 Measuring T cell activation

The enzyme-linked immunosorbent spot (ELISpot) assay was used to measure T cell activation after vaccination. The assay measures the frequency of cytokine-producing cells at a single cell level. In this study, we demonstrated increased IFN γ secretion following *in vitro* re-stimulation of splenocytes with recombinant HA from PR8, Cal07, H5N1, and H7N9. The splenocytes were harvested from mice vaccinated with the different vaccines encoding the HA¹ mosaic. Instead of complete HA proteins, we could have used individual peptides for stimulation of T cell responses. The use of peptides has both advantages and disadvantages. The particular peptide used will have a known binding preference for either MHC class I or class II molecules, thereby giving information about which T cells are activated. However, the selected peptide may not represent all epitopes, and may therefore give an incomplete detection of T cell activation.

4.1.5 Mice as an animal model for evaluation of influenza vaccines

Both ferrets and mice have played a central role in the development of vaccines and therapeutic drugs against influenza, and are still used in efforts towards the development of improved or even "universal" vaccines. Other animal models are available that can be used to better address the immunobiology of virus infection and the development of disease intervention strategies. These include: other rodents (guinea pig, hedgehog, hamster, and cotton rat), birds, swine, nonhuman primates (rhesus macaques, cynomolgus macaques, squirrel monkeys and others), and even humans[82]. In this study, *in vivo* experiments were

carried out in female BALB/c mice, a laboratory bred strain of albino mice that has proven useful for a variety of immunological studies. Also, the α MHCII-targeting unit used in some of the vaccine constructs is specific for the MHC class II molecules in BALB/c mice (I-Ed). The small size of mice, and the husbandry practice for mouse colonies, make them an affordable choice for animal model in influenza studies. One of their weaknesses, is that most influenza viruses do not naturally cause disease in mice[82]. Also, there are several metabolic, anatomic, and cellular differences between humans and mice, compared to the differences between a human and e.g. a rhesus macaque. A positive result in mice does therefore not necessarily mean a positive result in humans, but gives an important indication as to whether a vaccine is functional. Also, testing of a vaccine in mice never give a false positive – if it does not work in mice, it will not work in humans.

Mice are not natural hosts for influenza, but can nevertheless catch the disease if infected with some highly pathogenic viruses. The viral strains used for laboratory research typically have to be adapted to mice through serial passages. PR8 is an example of such a mouse-adapted strain, and has in the process lost the ability to infect humans. The virus is avirulent and can be administered intranasally in high doses[83]. Thus, it represents a safe animal model for influenza. Furthermore, it is easy to assess disease in infected animals. The clinical signs in mice include hunched posture, ruffled fur, weight loss, hind-limp paralysis, and death. Of these, weight loss can be more objectively assessed, and an ethically endpoint can be set well before the mouse dies. Here, we have used an endpoint of 20%. The relevance of weightloss as an indicator of disease has previously been verified by RT-PCR of viral loads in nasal washes, and by HE stainings of lung tissues harvested after viral challenge (Grødeland et al 2013[79]).

4.2 General discussion

4.2.1 Structure of the vaccine proteins

Sandwich ELISA assessed efficient secretion of the vaccine proteins. Previously, the targeting units of the vaccine proteins have been demonstrated functional by FACS analysis by binding to splenocytes or transfected cells (Grødeland et al 2013 [71]. However, the structural integrity of mosaic HA may only be assessed indirectly. Antibodies against HA (PR8 and

Cal07) failed to recognize mosaic HA antigens, and there are no available antibodies specific for the novel proteins that we have developed. In an attempt to visualize the vaccine proteins using SDS-PAGE and western blotting, an antibody specific for the dimerization domain of vaccibodies (HP6017-bio) was applied. Different conditions were used, including different gels (tris-glycine, bis-tris-glycine) and different gel concentrations (4-12%, 10%, 12%). Supernatants were up-concentrated prior to SDS-PAGE, in order to increase protein concentration. However, we were not able to obtain a functional western blot. We suggest that the concentration of the vaccine proteins in transfected 293E cells was too low for detection in the blot. Nevertheless, the vaccine proteins encoding mosaic HA protected mice against influenza challenges, and as such should bear structurally intact relevant antigenic structures.

4.2.2 Targeting units

Many molecules on APCs could be of interest for targeted vaccine delivery. In this study, we used targeting units that were already present in Bogen lab, and that previously had been shown to efficiently enhance immune responses. The chemokines MIP1 α and XCL1 are efficient recruiters of leukocytes, and may activate these cells to enhance the local inflammatory response. The co-expression of chemokines with antigen has previously been observed to increase immune responses[84, 85]. The chemokine MIP1 α binds the chemokine receptors CCR1, CCR3, and CCR5, which are members of the CC chemokine receptor family. The chemokine XCL1 mediates its chemotactic function by binding the chemokine receptor Xcr1, which is the only member of the C subfamily of chemokine receptors.

An scFv was applied for targeting of MHC class II molecules that are expressed on professional APCs and thymic epithelial cells. MCH class II molecules bind peptides from internalized pathogens and present them to CD4 T cells, which subsequently activate antibody production by B cells. Targeting with α MHCII has previously been shown to induce complete protection against influenza[79]. An scFv encoding the hapten NIP was utilized as a non-targeted control, and is of identical size and antigenic content as the scFv targeted to MHC class II molecules.

The efficacy of a single immunization with influenza HA targeted to MHC class II molecules, chemokine receptors (CCR) 1, 3, and 5, and Xcr1 has previously been evaluated[71, 79, 86]. All three targeting approaches induced HA-specific immune responses, and protected mice

against a lethal challenge with influenza virus, in contrast to a non-targeted control[71]. They did however, induce qualitatively different immune responses. One of the studies showed that targeting to MHC class II molecules predominantly induced an antibody/T_H2 response, whereas targeting to CCR1/3/5 or Xcr1 predominantly induced a CD8⁺/T_H1 T cell response[71]. This indicated that there is a receptor-dependent polarization of immune reactions towards either T_H1 or T_H2, indicating that we can tailor vaccines into giving a desired immune response against a pathogen[52]. Based on this, MIP1 α and XCL1 seem logical choices for targeting of the mosaic antigens, which were designed to activate T cell responses. In order to compare the CD8⁺/T_H1 targeting strategy with the antibody/T_H2 strategy, we employed α MHCII as a targeting unit as well.

4.2.3 Immune responses against HA

The ELISA assays performed for measurement of induced antibodies, and the ELISpot for detection of activated T cells, tells us about the amount of induced immune responses, but not about how functional they are in preventing influenza.

The findings of Grødeland et.al. (Grødeland 2015) demonstrated that targeting to Xcr1 leads to a low induction of IgG1 and a high induction of IgG2a. Targeting with MIP1 α induces a similar result, and targeting with α MHCII induces clearly more IgG1 than IgG2. Targeting with the chemokines MIP1 α or XCL1 will lead to activation of T_H1 cells (and CD8 T cells), while targeting with α MHCII will lead to activation of T_H2 cells. From theory, we know that a T_H1 response is associated with the induction of IgG2a, IgG2b, and IgG3, and that T_H2 is associated with the induction of IgG1[79, 87]. In this study, we detected antibodies using total IgG, meaning all different IgG isotypes will be detected. Therefore we can not say anything about the types of IgG induced. Serum antibodies against HA is a well-established correlate of protection against influenza[88, 89], and antibodies are the means of protection for the currently used inactivated trivalent influenza. Regarding activation of T cells, XCL1 and MIP1 α have been demonstrated to be the most effective targeting units for induction of CD8 T cell protection. This has been shown by depletion assays where CD8 T cells have been depleted and protection followingly abolished. Depletion of CD8 and CD4 T cells using using MHCII targeting, did however not abolish protection[71].

Antigen targeting with MIP1 α has previously been shown to induce both T cell responses and antibodies[52, 71]. Here, we found little antibodies when examining the induced responses

against PR8, but substantially more when examining antibody responses against HAs from Cal07 and H5. Interestingly, despite the very low amount of antibody responses detected against PR8, both MIP1 α -HA¹ and MIP1 α -HA¹⁻¹⁷ could provide protection in a viral challenge with PR8. In fact, when comparing to the other mosaic vaccines, targeting of mosaic HAs to chemokine receptors with MIP1 α as targeting unit provided better protection against PR8 both in terms of reduced weight loss and survival proportions. These results indicate that MIP1 α -targeting of the mosaics could induce both T cells and antibodies, which correlates with previous studies. Based on the findings of Grødeland et.al. (Grødeland 2015), we can assume that the induced antibodies are of the IgG2a isotype, due to activation of T_H1 helper cells. We did not test whether the activated T cells were CD4 T cells or CD8 T cells, but from previous studies, we know that antigenic peptides are presented on both class I and class II molecules when targeted with chemokines like MIP1 α (or XCL1). We could have tested this by doing depletions of one of the T cell types. The dual presentation of mosaic antigen on the same APC has been described important for efficient activation of CD4 T helper cells that can augment the formation of cytotoxic T cells[54].

Targeting of antigen using XCL1 has been demonstrated to almost exclusively induce T cell responses[52]. This correlates well with the low titers of antibodies induced with the HA¹ mosaic vaccines against all three different HAs. An exception was the quite high titers against HA of Cal07 after intradermal vaccination, which might have contributed to the protection against Cal07. This might also explain why the observed protection was better against Cal07 than PR8. In contrast to MIP1 α -HA¹, MIP1 α -HA¹⁻¹⁷ induced some antibodies against H5 and Cal07. However, based on previous results[86], we speculate that the observed protection against influenza challenges is due to the activation of T cells. XCL1 activated the highest amount of T cells in the ELISpot, which is in line with the findings of Fossum et.al. (Fossum 2015); that targeting of the vaccines to Xcr1, predominantly leads to presentation on MHC class I molecules that interact with CD8 T cells.

Targeting of the HA¹ mosaic towards MHC class II molecules led to poor induction of antibodies following both intradermal and intramuscular vaccination. This is in contradiction with previous results from influenza vaccine experiments, which have shown that targeting with an scFv specific for MHC class II molecules can induce high titers of neutralizing antibodies against the HA antigen. Also, it is the only targeting unit able to mediate a

complete antibody-mediated protection against influenza (Grødeland et.al., 2013). In contrast, we here observed a complete lack of antibodies against all three viruses used in the ELISA assays. An explanation for this could be that the design of the mosaic HA (with 9-mers) have removed the relevant peptides that could be presented on MHC class II molecules for activation of CD4 helper cells, and the later activation of B and plasma cells. However, it can also not be ruled out that there is an undiscovered flaw in the particular MegaPrep used for vaccinations. An argument indicating this is the observed antibody responses after vaccination with α MHCII-HA¹⁻¹⁷, as opposed to α MHCII-HA¹. In correspondence with the lack of induced antibodies after vaccination with MHCII-HA¹, this vaccine did not confer protection against PR8, and only one of the mice survived in the challenge with Cal07. Vaccination with α MHCII-HA¹⁻¹⁷ did not induce production of antibodies against PR8, either, but induced antibodies against Cal07 and H5. In the challenge experiment with HA¹⁻¹⁷, some protection against influenza PR8 was observed.

In contrast to the challenge experiments with the HA¹ mosaic, we saw a clear targeting effect in the experiment with the HA¹⁻¹⁷ mosaic. All except one NIP-mice died, and the other three targeted mosaic vaccines, especially MIP-HA¹⁻¹⁷, gave a higher survival and protection overall. Some targeting effect was seen in the challenge with PR8 and the HA¹ mosaic, as MIP1 α led to a higher survival rate than α NIP. XCL1 gave a similar survival rate as α NIP, but the XCL1 mice gained weight faster than the α NIP mice. Overall, MIP1 α conferred the best protection against both PR8 and Cal07.

4.2.4 Why the mosaic approach give a broader protection

The measured T cell and antibody responses, and the data from the viral challenges in this study, indicate a broader immunogenicity of the mosaic HAs as compared to a natural HA sequence. This is in line with previous studies[2, 69, 90]. One mechanism that might explain the breadth of protection, is that the 9-mer mosaics capture more T cell epitopes than a natural HA sequence. This could predominantly have led to a higher level of activated CD8 T cells, which epitope length are most commonly 9 amino acids long, but also to activation of CD4 T cells. Activation of CD4 T cells will have led to antibody responses broader than those inferred by a natural HA, through activation of B cells by T_H1 helper cells. A second mechanism that might explain the breadth of protection, is that the mosaics have maintained an intact antigenic structure, and thereby its physiological function. Presumably, this will

make the mosaic proteins mimic natural processing, so that epitopes that stimulate T-cell responses in the vaccine will be the same epitopes that are processed and presented in natural influenza infection. Similarities between the virus and vaccine antigen processing could enhance the likelihood that the vaccine stimulates T cells that recognize virus epitopes presented by infected cells and that are not vaccine specific. Also, as the mosaics have a normal hemagglutinin function, they are presumably expressed as a cleavable protein. This might enable the stimulation of neutralizing antibody responses against the stem of HA. As our mosaics contain the most common stalk epitopes, the stem-specific antibodies could possibly be more cross-protective.

4.2.5 Future perspectives

Of the two mosaic antigens, the results with the HA¹⁻¹⁷ mosaic was the most promising. HA¹⁻¹⁷ induced the overall highest antibody titers against the three HAs, and it also mediated the best protection against PR8. These results indicate that the HA¹⁻¹⁷ mosaic is the most cross-protective antigen of the two mosaics. Although it is the least similar sequence to PR8, it conferred a better protection than HA¹ in the viral challenge. The challenge with HA¹⁻¹⁷ also showed a clear targeting effect, where the non-targeted control was the least protective vaccine against PR8, as predicted from previous studies with vaccibodies. However, other immunological aspects of the vaccine constructs need to be further characterized. Conducting an ELISpot assay for the HA¹⁻¹⁷ mosaic constructs will test the ability of the mosaic to activate T cells. For an assessment of T cell contributions to protection, a T cell depletion can be conducted, with depletion antibodies against CD8 T cells, CD4 T cells, or both. Cytokine profiles, antibody epitope coverage, and mapping, are other aspects that can be investigated in order to fully understand the mechanism responsible for protection. Furthermore, it would be interesting to create another HA¹⁻¹⁷ mosaic with an epitope length set to 12 amino acids. This change in epitope length might increase the ability of the mosaic to capture CD4 T cells epitopes, and a comparison of induced antibody titers could be performed.

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Appendix 1: Abbreviations

aa	amino acids
Ab	antibody
APC	antigen presenting cell
BCR	B- cell receptor
BSA	bovine serum albumin
Cal07	A/California/07/2009
DNA	deoxyrinucleic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunosorbent spot assay
FCS	fetal calf serum
HA	hemagglutinin
HEK	human embryonic kidney
HI	hemagglutinin inhibition
HRP	horse radish peroxidase
IFN	interferon
Ig	immunoglobulin
ITAM	immunoreceptor tyrosine-based activation motif
MHC	major histocompatibility complex
NA	neuraminidase
NaCl	sodium chloride
NIP	4-hydroxy-3-iodo-5-nitrophenylacetyl
NP	nucleoprotein
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PCR	poly chain reaction
PR8	influenza A/PR8/8/34 (H1N1)
PRR	pattern recognizing receptors
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid

RPMI	Roswell Park Memorial Institute
RT	room temperature
scFv	single chain fragment variable
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard deviation of mean
SN	supernatant
TAE	tris acetate EDTA buffer
TCR	T cell receptor
Th1	T helper 1
Th2	T helper 2
UV	ultraviolet

Appendix 2: Supplementary protocols

Preparation of agarose gels:

- Measure 1 g of agarose powder and add it to a microwaveable flask.
- Add 50 ml of 1xTAE buffer and swirl the flask.
- Heat the solution in a microwave for several short intervals until the agarose is dissolved – do not let the solution boil for longer periods as it may boil out of the flask.
- Let the solution cool to about 50-55°C, swirling the flask occasionally.
- Add 4 µl of a SYBR safe gel stain (ThermoFischer) that will bind to nucleic acids and allow the DNA to be visualized in ultraviolet (UV) light.
- Pour the agarose solution into a casting tray and place a comb in the appropriate place.
- Let the gel solidify in room temperature for 20-30 minutes.
- Carefully pull out the comb and place the gel in the electrophoresis chamber (Bio-Rad).
- Add enough 1xTAE buffer to completely cover the gel.

Procedure for agarose gel electrophoresis:

- Add 4 µl of Sample Loading Buffer to each sample and mix.
- Carefully pipette 20 µl of each sample into separate wells on the gel.
- Pipette 10 µl of a 1 kb DNA ladder (BioNordika, NEB) and 10 µl of 100 bp DNA ladder (BioNordika, NEB) as molecular weight standards into a well each.
- Run the gel at 100 V until dye has moved approximately 75-80% down the gel.

Preparing single-cell suspensions for ELISpot

- Anaesthetize mice by i.p. injection of 150 µl ZRF and euthanize by cervical dislocation
- Lay mice on dissecting board, "left side" uppermost.
- Surface-sterilize the skin using 70% ethanol.
- Using sterile surgical instruments (scissors and forceps), cut through the skin just below the ribcage to visualize the spleen.
- Remove the spleen with a smaller set of sterile instruments and trim away any fatty tissue.
- Place spleen in a 15 ml tube containing RPMI medium with 10% FCS and supplements (ThermoFischer) and keep on ice for the whole procedure.
- Sterilize a cell-dissociation sieve and a glass pestle with 70% alcohol.
- Strain cells by pouring the spleen and medium through a sterile cell-strainer and into a Petri dish.
- Press the spleen through the strainer using the sterile glass pestle.
- Transfer spleen cell suspension into 15 ml tube and keep on ice.
- Repeat steps 1-4 for all tubes containing the harvested spleens.

Appendix 3: Recipes

Buffers for ELISA assays:

Blocking buffer	300 ml
30% BSA	10 ml
PBS azide (0,05%)	300 ml
ELISA buffer	500 ml
30% BSA	1,66 ml
Tween 20	1,0 ml
PBS azide (0,05%)	497,3 ml
Substrate buffer	1 liter
Diethanolamin	97 ml
dH ₂ O	800 ml
MgCl ₂ x 6 H ₂ O (Mm = 203,3027 g/mol)	101 mg
Azid (NaN ₂) (51,0032 g/mol)	200 mg
ELISA wash buffer	10 liters
NaH ₂ PO ₄ xH ₂ O (Mm = 119,9770 g/mol)	44,8 g
Na ₂ HPO ₄ x12H ₂ O (Mm = 141,9588 g/mol)	483,5 g
NaCl (Mm = 58,44 g/mol)	2045 g
Tween 20	250 ml
dH ₂ O	10 L

Appendix 4: Supplementary results, figures, and tables

Mosaic hemagglutinin sequences (amino acids)

HA¹:

DTICIGYHANNSTDTVDTVLEKNVTVTHSVNLLED SHNGKLC LLKGIAPLQLGKCNIAGW
LLGNPECDLLL TANSWSYIETSNSENGTCYPGEFIDYEELREQLSSVSSFERFEIFPKT
SSWPNHETTKGVTAACSYSGASSFYRNLLWITKKGTSYPKLSKSYTNNKGKEVLVLWGVH
HPPNIGDQRALYHTENAYVSVVSSHYSRRFTPEIAARPKVRGQAGRMNYYWTLLDQGDIT
TFEATGNLIAPWYAFALSRGFGSGIITSNAPMDECDAKCQTPQGAINSSLPFQNVHPVTI
GECPKYVKSTKLRMATGLRNIPSIQSRGLFGAIAAGFIEGGWTGMIDGWYGYHHQNEQSG
YAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGFLDIWT
YNAELLV LLENERTLDFHDSNVKNLYEKVKSQLKNNAKEIGNGCFEFYHKCDDECMESVK
NGTYDYPKYSEESKLNREKIDGVKLESMGVYQILAIYSTVASSL

HA¹⁻¹⁷:

DKICLGHHAVANGTKVNTLTERGVEVVNATETVERTNVPRICSKGKRTVDLGQCGLLGT
IGPPQCDQFLEFSADLIERREGSDVCYPGKFTNEESLRQILRESGGIDKESMGFTYSGI
RTNGATSACRRSGSSFYAEMKWLLSNSDNAAFPQMTKSYRNPRNKPALIIWGIHHSSTT
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Mosaic hemagglutinin sequences (nucleotides)

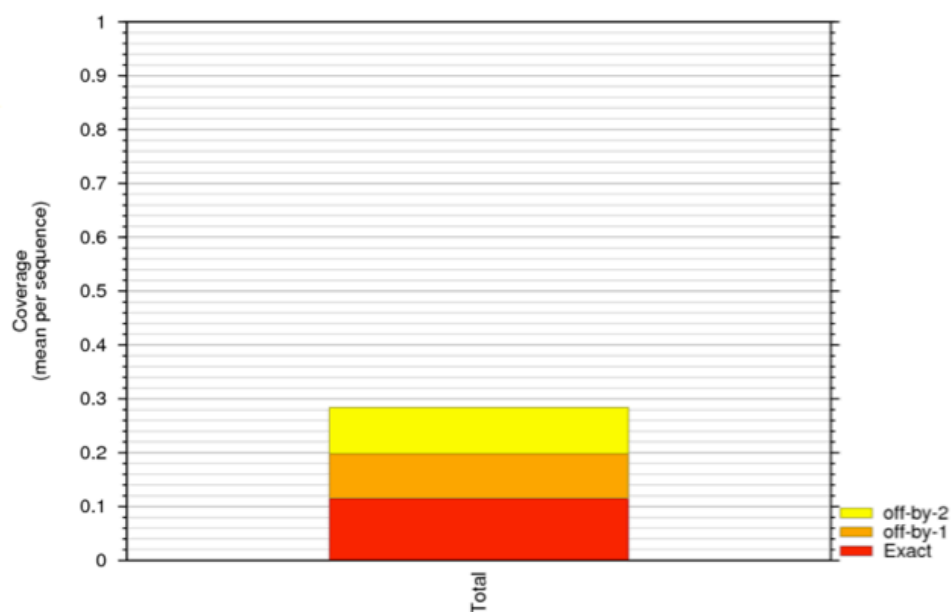
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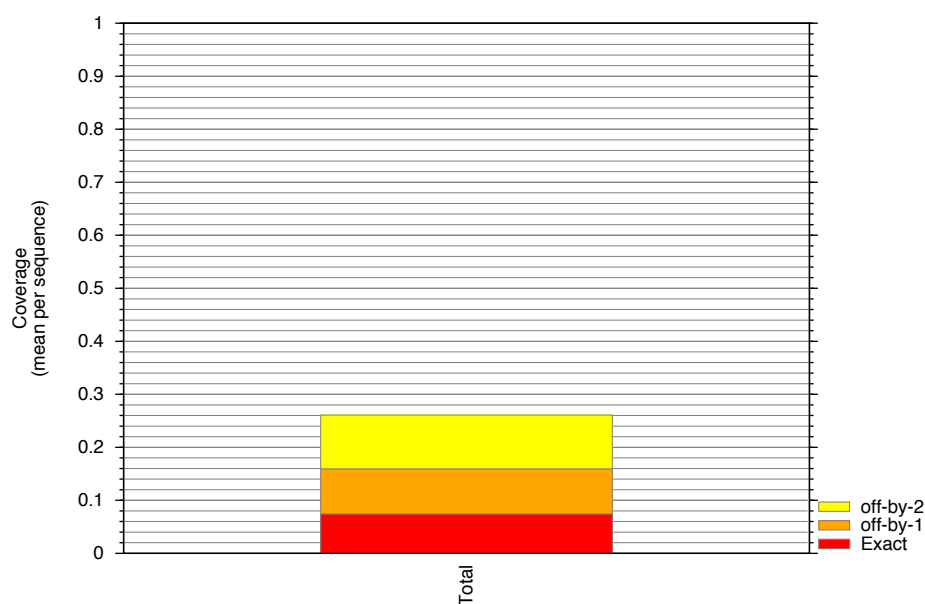
HA¹⁻¹⁷:

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Epitope coverage



Supplementary figure 1. Epitope coverage of HA¹⁻¹⁷ : The mean fraction of natural-sequence 9-mers shared with HA¹⁻¹⁷ averaged over all sequences in the HA¹⁻¹⁷ input set. Both exact matches (red), off-by-one matches (orange) and off-by-2 matches (yellow) were included in the score.



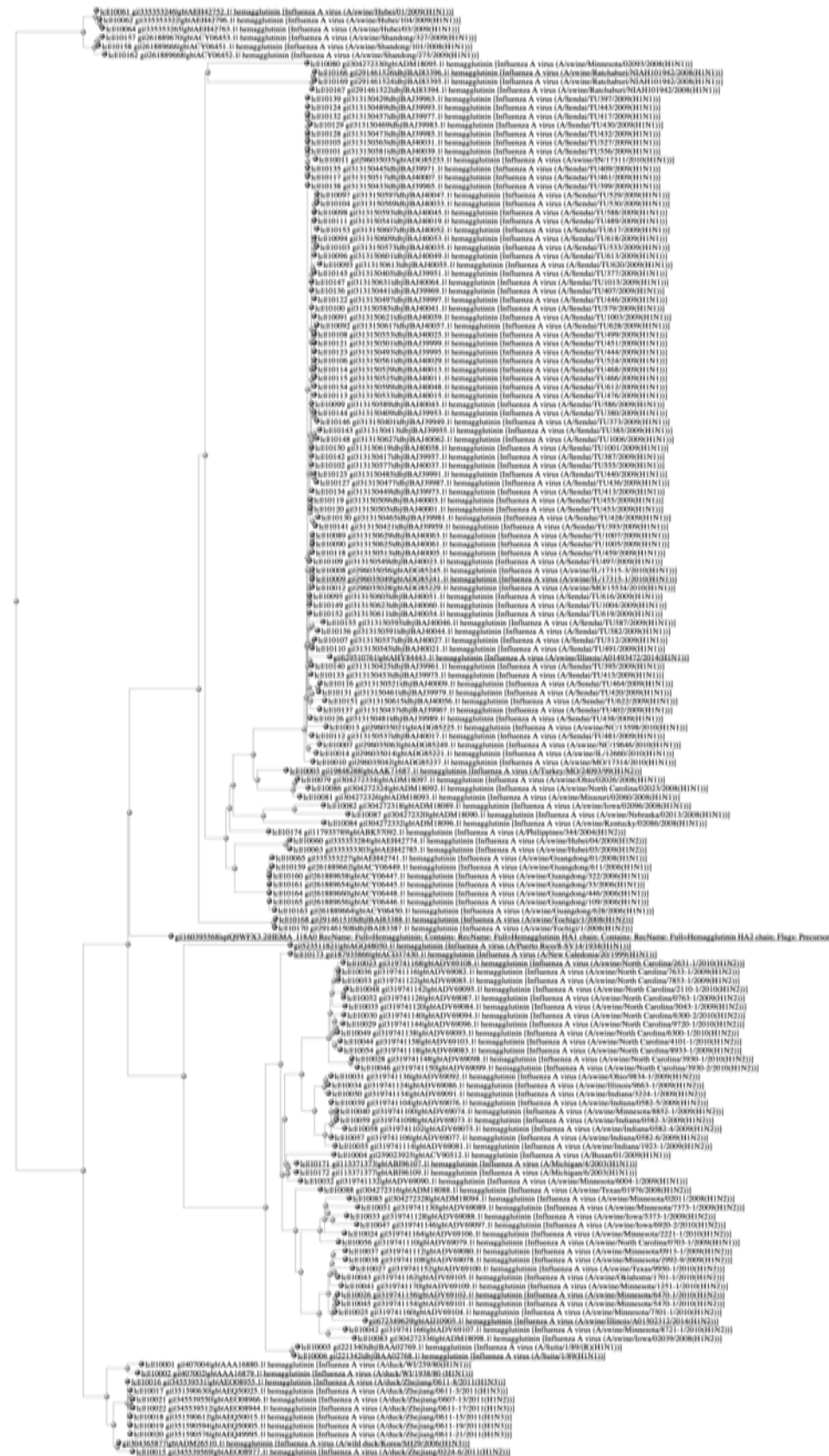
Supplementary figure 2: Epitope coverage of HA (PR8): The mean fraction of natural-sequence 9-mers shared with H1 (PR8) averaged over all sequences in the HA¹⁻¹⁷ input set. Both exact matches (red), off-by-one matches (orange) and off-by-2 matches (yellow) were included in the score.

Supplementary tables

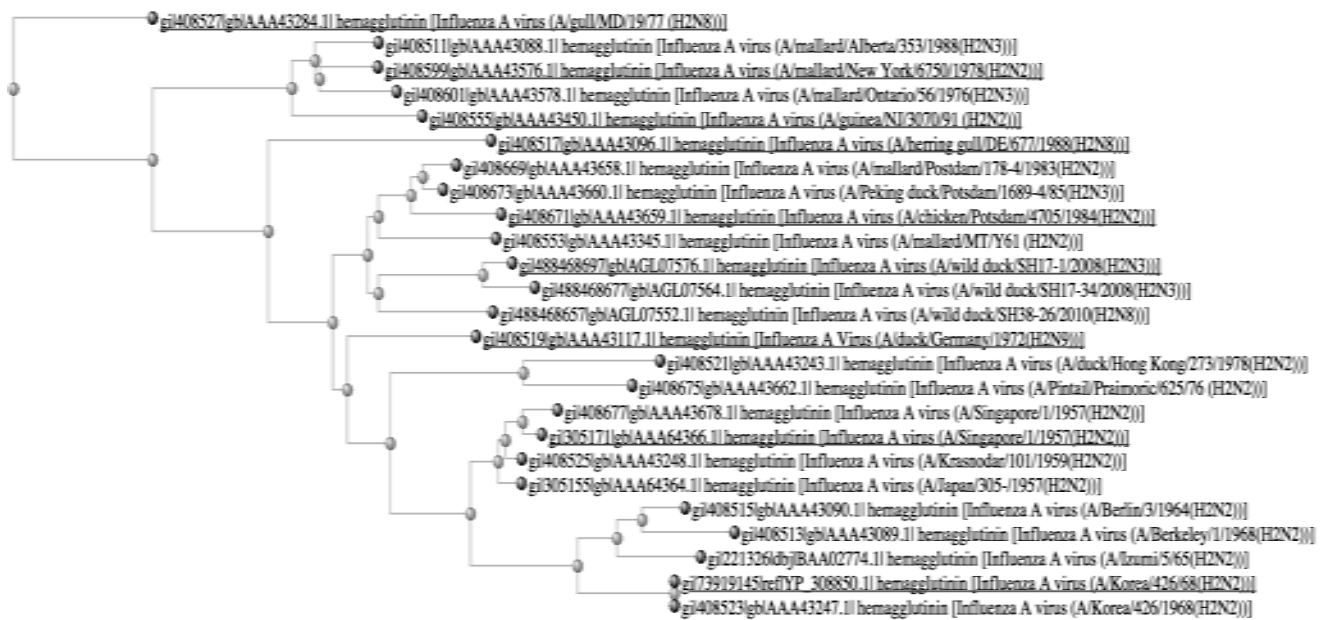
Supplementary table 1: Sequences selected for generation of the HA¹⁻¹⁷ mosaic

HA subtype	Number of input sequences
H1	18
H2	9
H3	9
H4	8
H5	8
H6	8
H7	12
H8	1
H9	9
H10	8
H11	8
H12	1
H13	2
H14	2
H15	3
H16	5
H17	2
Total	112

Phylogenetic trees



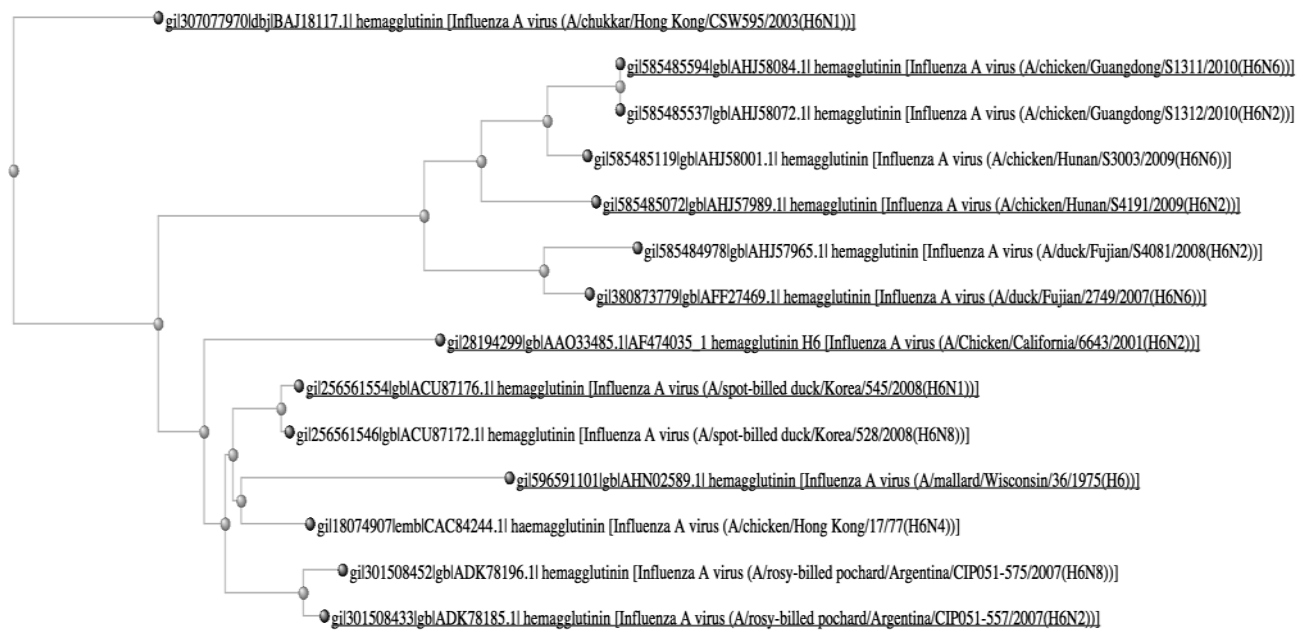
Supplementary figure 3: Phylogenetic tree of collected H1 sequences



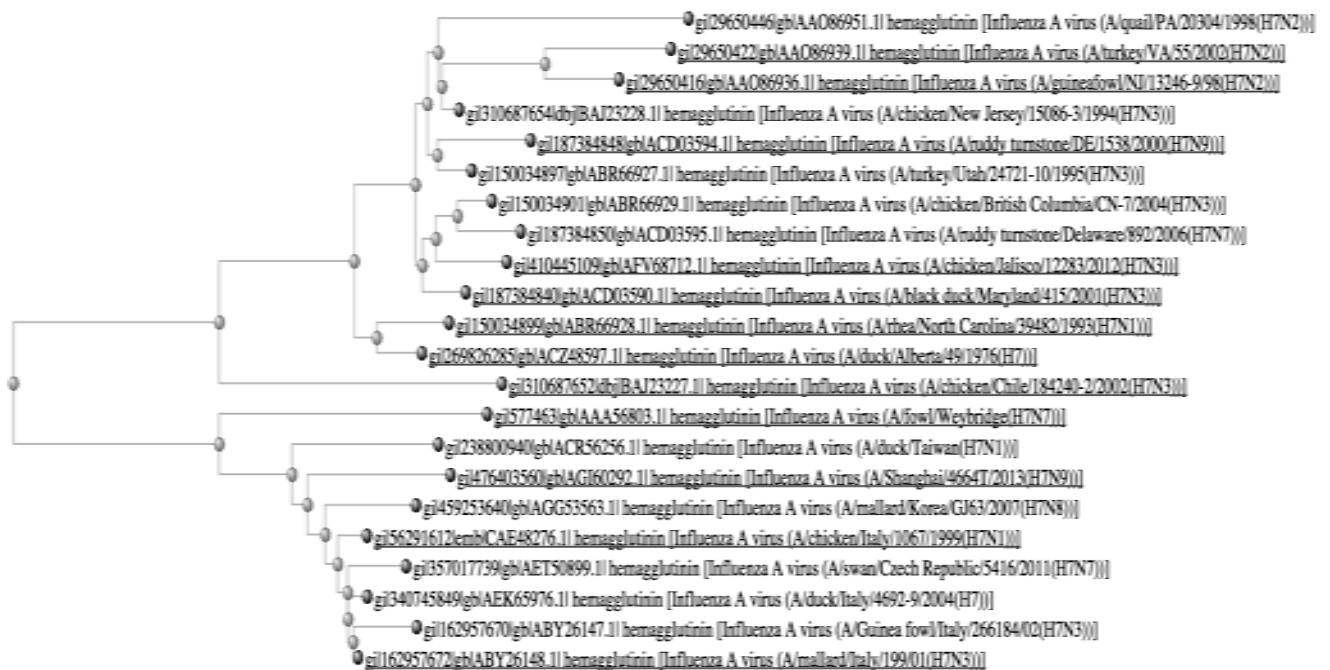
Supplementary figure 4: Phylogenetic tree of collected H2 sequences



Supplementary figure 5: Phylogenetic tree of collected H4 sequences



Supplementary figure 6: Phylogenetic tree of collected H6 sequences



Supplementary figure 7: Phylogenetic tree of collected H7 sequences



Supplementary figure 8: Phylogenetic tree of collected H9 sequences